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Polydrug Opioid Abuse & Mechanisms of Tolerance to Opioid Respiratory Depression

Polydrug Opioid Abuse & Mechanisms of Tolerance to Opioid Respiratory Depression

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Abstract

The primary cause of fatality in an opioid overdose is opioid induced respiratory depression. In addition to using opioids, polydrug use is common within the opioid using population, with ethanol being the most common additional drug of abuse, though another emerging class of co-abused drugs is the gabapentoids, including pregabalin. Ethanol has previously been shown to reverse morphine but not methadone induced tolerance to morphine respiratory depression, suggesting ethanol intrinsically interacts with maintained tolerance at the μ-opioid receptor. This thesis aimed to investigate the development of tolerance to abused opioids, the interaction of ethanol and pregabalin with opioid tolerance, and the potential molecular mechanisms required to maintain opioid tolerance.

Morphine, oxycodone, methadone and fentanyl all dose-dependently depressed respiration. Oxycodone did not display any contribution from δ- or κ-opioid receptors to its respiratory depressant effect. Fentanyl respiratory depression however, was significantly harder to antagonise than morphine respiratory depression, with a 10-fold greater dose of naloxone required, suggesting that the increase in fentanyl overdose deaths requires a re-assessment of first responder guidelines regarding naloxone administration to rescue respiration.

Prolonged treatment of mice with morphine, oxycodone and methadone were all able to induce tolerance to morphine respiratory depression. Morphine as the primary metabolite of heroin is likely to interact with both oxycodone and methadone induced tolerance suggesting cross-tolerance between abused opioids will offer some protection from overdose in humans.

Morphine and oxycodone tolerance were reversed by ethanol, pregabalin and PKC inhibition, suggesting commonality in the mechanisms by which these agonists induce tolerance. Methadone was contrarily not reversed by any of these treatments. Acute fentanyl tolerance was however reversed by GRK inhibition, indicating a potential delineation in mechanisms of tolerance dependent on agonist efficacy; with lower efficacy agonist predisposed toward G-protein dependent tolerance and higher efficacy agonist predisposed toward GRK dependent tolerance.

This thesis provides evidence for the continued necessity of understanding the drugs of concern in opioid polydrug abuse and assessing the reasons for enhanced comorbidity, beyond assuming summation of effect. Additionally, this thesis proposes PKC as the primary mediator of tolerance for both morphine and oxycodone tolerance to respiratory depression with fentanyl tolerance mediated by GRK. Further elucidation of polydrug abuse and mechanisms of tolerance will allow better care for opioid prescribed patients and opioid abusers, as well paving the way for the development of better opioid analgesics.

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"*Fortitudine Vincimus* – By endurance we conquer"

Author’s Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: ..... DATE:.....

Table of Contents

Abstract..... i

Acknowledgements..... ii

Author's Declaration..... iii

Table of Contents..... iv

Table of Figures..... xiv

Table of Tables..... xviii

Table of Appendices..... xix

Table of Abbreviations..... xx

CHAPTER 1 General Introduction..... 1

1.1 A Brief History of Opioid Abuse..... 1

1.2 Abused Opioids: Heroin, Methadone, Oxycodone and Fentanyl..... 2

1.2.1 Heroin..... 2

1.2.1.1 Heroin Metabolism and Pharmacology..... 3

1.2.2 Methadone..... 4

1.2.2.1 Methadone Metabolism and Pharmacology..... 4

1.2.3 Oxycodone..... 5

1.2.3.1 Oxycodone Metabolism and Pharmacology..... 5

1.2.4 Fentanyl..... 6

1.2.4.1 Fentanyl Metabolism and Pharmacology..... 6

1.3 Opioid Receptor Activation..... 7

1.4 μ-opioid Receptor Location..... 9

1.5 μ-opioid Receptor Desensitization and its Relation to Tolerance..... 9

1.6 Phosphorylation of the MOPr by GRK and PKC..... 11

1.7 Desensitization and Internalisation of the MOPr..... 13

1.8 Respiratory Control and Opioids..... 14

1.9 Tolerance to Respiratory Depression..... 16

1.9.1 Convergence of Tolerance to Opioid Respiratory Depression and Euphoria……..17

1.10 Ethanol and Opioids……19
1.10.1 Aetiological Evidence of Ethanol and Opioids……19
1.10.2 Social Context and Opioid Overdose……21
1.10.3 Opioid Induced Desensitization and Ethanol in Brain Slices……21
1.10.4 Morphine Antinociception in Mice and Ethanol……23
1.10.5 Morphine Respiratory Depression in Mice and Ethanol……23
1.11 Alcohol in Society……25
1.12 Ethanol Pharmacology……26
1.12.1 Ethanol Metabolites…… 28
1.13 The Emerging Problem of Gabapentoids and Opioids……29
1.13.1 Gabapentoid Pharmacology…… 29
1.14 Protein Kinase C and Morphine……30
1.14.1 Electrophysiological Evidence……30
1.14.2 *In vivo* Evidence……32
1.15 The Role of PKC and Ethanol in Opioid Tolerance……34
1.16 Intrinsic Agonist Efficacy for G-protein – GRK Signalling: A Predictor of Susceptibility to Reversal by Ethanol……34
1.17 Hypotheses……35

CHAPTER 2 Materials and Methods…….. 36

2.1 Ethical Consideration…….. 36
2.2 Animals…… 36
2.3 Drugs…… 36
2.4 Induction of Opioid Tolerance…… 37

2.4.1 Implantation of Prolonged Opioid Delivery Systems.....37
2.4.1.1 Implantation of Morphine Pellet..... 37
2.4.1.2 Implantation of Osmotic Mini-pumps for Prolonged Opioid Administration.....37
2.4.1.3 Pump Preparation.....38
2.4.1.4 Morphine Pump Protocol.....38
2.4.1.5 Oxycodone Pump Protocols.....38
2.4.1.6 Methadone Pump Protocol.....38
2.4.2 Induction by Multiple Injections Protocol.....39
2.4.2.1 Multiple Morphine Injections.....39
2.4.2.2 Multiple Fentanyl Injections.....39
2.5 Surgical Procedure.....39
2.6 Monitoring Respiration in Freely Moving Mice.....40
2.7 Analysis of Respiratory Waveforms.....42
2.8 Respiration Experiments..... 43
2.8.1 Measurement of Acute Opioid Respiratory Depression.....43
2.8.2 Measuring the Onset of Tolerance to Opioid Respiratory Depression.....43
2.8.3 Measuring Tolerance to Acute Opioid Respiratory Depression.....44
2.8.4 Measurement of Reversal of Tolerance to Opioid Respiratory Depression.....44
2.9 Analysis of Respiratory Data.....45
2.9.1 Example of Percent of Baseline Minute Volume Calculation.....45
2.9.2 Analysis of Percent of Baseline Minute Volume Data.....46
2.10 Measurement of Nociceptive Reflex.....47
2.11 Nociception Experiments.....48
2.11.1 Acute Antinociception.....48
2.11.2 Tolerance to Opioid Antinociception.....48
2.11.3 Reversal of Tolerance to Opioid Antinociception.....48
2.11.4 Acute Antinociception Tolerance..... 49
2.12 Measurement of Both Opioid Induced Antinociception and Respiratory Depression..... 50

2.13 Prolonged Ethanol Diet Administration.....51
2.13.1 Diet Constitution..... 51
2.13.2 Experimental Protocol.....52
2.13.3 Mouse Welfare.....53
2.13.4 Induction of Tolerance and Measurement of Respiratory Depression.....54
2.13.5 Sampling of Mouse Blood and Brain..... 55
2.14 Preparation of Plasma and Brain Samples for Morphine Content Analysis..... 56
2.14.1 Plasma Sample Preparation..... 56
2.14.2 Brain Sample Preparation..... 56
2.14.3 Morphine Extraction from Plasma and Brain Samples..... 56
2.15 Preparation of Plasma Samples for Ethanol Content Analysis.....59
2.15.1 Plasma Sample Preparation.....59
2.15.2 Plasma Sample Spiking..... 59
2.15.3 Analysis of Plasma and Brain Samples for Morphine Content..... 60
2.15.4 Analysis of Plasma Samples for Ethanol Content..... 60
2.16 Experimental Design.....61
2.16.1 Randomisation and Blinding..... 61
2.16.2 Statistical Power Analysis..... 61
2.16.2.1 Maximal Effect Size.....62
2.16.2.2 Minimal Effect Size..... 62
2.17 Data Analysis..... 63
2.17.1 One-way ANOVA..... 63
2.17.2 Two-way ANOVA..... 63
2.17.3 Post Test Choice.....64

CHAPTER 3 Acute Effect of Opioids..... 65

3.1 Introduction.....65
3.1.1 Chapter Aims.....65
3.2 Morphine and Oxycodone.....66
3.2.1 Effect of Morphine on Respiration.....66

3.2.2 Baseline Respiration and Correlation with Maximum Respiratory Depression..... 68
3.2.3 Effect of Morphine on Nociception.....68
3.2.4 Effect of Oxycodone on Respiration.....70
3.2.5 Effect of Oxycodone on Nociception.....72
3.2.6 Receptor Activity of Oxycodone and Morphine.....72
3.2.7 Oxycodone Agonist Activity at κ-Opioid Receptors.....72
3.2.8 Oxycodone Agonist Activity at δ-Opioid Receptors.....74
3.2.9 Nor-BNI Antagonism of the Specific κ-Opioid Agonist U69,593.....74
3.2.10 Morphine Agonist Activity at δ- / κ-Opioid Receptors.....76

3.3 Methadone.....78
3.3.1 Effect of Methadone on Respiration.....78
3.3.2 Effect of Methadone on Nociception.....81
3.4 Fentanyl.....82
3.4.1 Effect of Fentanyl on Respiration.....82
3.4.2 Naloxone Reversal of Fentanyl Respiratory Depression.....85
3.4.3 Effect of Fentanyl on Nociception.....89
3.5 Discussion.....90
3.5.1 Relative Potency of Opioid Agonists.....90
3.5.2 Opioid Receptor Agonism by Oxycodone.....92
3.5.3 Fentanyl Resistance to Naloxone Antagonism.....92

CHAPTER 4 Development of Tolerance to Opioids.....93

4.1 Introduction.....93
4.1.1 Chapter Aims.....94
4.2 Morphine Induced Tolerance to Morphine.....95
4.2.1 Acute Tolerance to Morphine.....95
4.2.2 Development of Tolerance with Twice Daily Doses of Morphine.....95
4.2.3 Development of Tolerance with Continuous Exposure to Morphine Through an Osmotic Mini-Pump.....98

4.2.4 Tolerance to Morphine Induced by Subcutaneous Morphine Pellet Implantation.....100
4.3 Development of Tolerance to Morphine with Continuous Exposure to Oxycodone.....102
4.4 Development of Tolerance to Morphine with Continuous Exposure to Methadone.....108
4.5 Acute Tolerance to Fentanyl.....110
4.6 Discussion.....112
4.6.1 Morphine Tolerance.....112
4.6.2 Oxycodone Tolerance.....113
4.6.3 Methadone Tolerance.....114
4.6.4 Fentanyl Tolerance.....114
4.6.5 Overall Conclusion.....115
Chapter 5 Mechanisms of Opioid Tolerance.....116
5.1. Introduction
5.1.1. Protein Kinase C.....116
5.1.2. c-Jun N-terminal Kinase.....117
5.1.3. G-protein Receptor Kinase.....117
5.1.4. Chapter Aims.....118
5.2. Morphine.....119
5.2.1. Effect of Tamoxifen on Tolerance Induced by Prolonged Morphine Treatment..... 119
5.2.2. Effect of Calphostin C on Tolerance Induced by Prolonged Morphine Treatment..... 121
5.2.3. Effect of Prolonged Morphine Treatment in PKCα Knock-out Mice.....123
5.2.4. Effect of SP600125 on Tolerance Induced by Prolonged Morphine.....125
5.3. Oxycodone..... 127
5.3.1. Effect of Calphostin C on Medium Oxycodone Induced Morphine Tolerance.....127

5.3.2. Effect of Calphostin C on High Oxycodone Induced Morphine Tolerance.....129
5.4. Methadone.....131
5.4.1. Effect of Tamoxifen and Calphostin C on Prolonged Methadone Induced Morphine Tolerance..... 131
5.5. Fentanyl.....133
5.5.1. Effect of Compound 101 on Acute Tolerance to Fentanyl Antinociception.....133
5.5.2. Effect of Compound 101 on Acute Tolerance to Fentanyl Respiratory Depression.....135
5.5.3. Effect of Calphostin C on Acute Tolerance to Fentanyl Antinociception.....137
5.5.4. Effect of Calphostin C on Acute Tolerance to Fentanyl Respiratory Depression..... 139
5.6. Discussion.....140
5.6.1. Morphine.....140
5.6.1.1. Protein Kinase C..... 140
5.6.1.2. c-Jun N-terminal Kinase.....141
5.6.1.3. Conclusion.....142
5.6.2. Oxycodone..... 142
5.6.3. Methadone.....143
5.6.4. Fentanyl.....144
5.6.5. Overall Conclusion.....145

Chapter 6 Opioid Tolerance and Polydrug Abuse..... 146

6.1. Introduction..... 146

6.1.1. Ethanol..... 146

6.1.2. Acetaldehyde..... 147

6.1.3. Pregabalin..... 148

6.1.4. Chapter Aims.....148

6.2. Acute Ethanol..... 149

6.2.1. Effect of Acute Ethanol on Morphine Induced Tolerance to Morphine Respiratory Depression..... 149

6.2.2. Effect of Acute Ethanol on Medium Oxycodone Induced Tolerance to Morphine Respiratory Depression.....152

6.2.3. Effect of Acute Ethanol on High Oxycodone Induced Tolerance to Morphine Respiratory Depression.....155

6.3. Effect of Prolonged Ethanol Diet on Morphine Induced Tolerance to Morphine Respiratory Depression..... 158

6.3.1. Consumption of Control and Ethanol Diet.....158

6.3.2. Mouse Respiration During Morphine Tolerance Induction.....160

6.3.3. Effect of Ethanol Diet on the Expression of Tolerance to Morphine Respiratory Depression..... 162

6.3.4. Effect of Ethanol Diet on Morphine Distribution.....164

6.4. Effect of Acute Acetaldehyde on Morphine Induced Tolerance to Morphine Respiratory Depression..... 166

6.4.1. Morphine Pellet Induced Tolerance

6.4.2. Effect of Acetaldehyde on Morphine Pellet Induced Tolerance.....168

6.5. Acute Pregabalin

6.5.1. Effect of Acute Pregabalin on Morphine Induced Tolerance to Morphine Respiratory Depression..... 171

6.5.2. Effect of Acute Pregabalin on Medium Oxycodone Induced Tolerance to Morphine Respiratory Depression.....174

6.5.3. Effect of Acute Pregabalin on High Oxycodone Induced Tolerance to Morphine Respiratory Depression....176
6.5.4. Effect of Acute Pregabalin on Methadone Induced Tolerance to Morphine Respiratory Depression....179
6.6. Discussion....181
6.6.1. Ethanol....181
6.6.2. Acetaldehyde....183
6.6.3. Prolonged Ethanol Consumption....184
6.6.4. Pregabalin....185
6.6.5. Conclusion....185

Chapter 7 General Discussion....186

7.1. Introduction....186
7.2. Tolerance Develops to Opioid Respiratory Depression....187
7.3. Methadone Induces Tolerance to Morphine Respiratory Depression....189
7.4. Oxycodone Induces Tolerance to Morphine Respiratory Depression....190
7.5. Tolerance to OIRD as a Consequence of the Experimental Protocol Used....192
7.6. Opioids and Polydrug Abuse: Ethanol, Cocaine and Benzodiazepines....194
7.7. Ethanol Reversal of Tolerance....195
7.8. PKC Inhibition Reversal of Tolerance....196
7.9. Ethanol and the Inhibition of PKC....197
7.10. Gabapentoid and Opioid Abuse....199
7.11. G-protein Vs GRK/Arrestin Signalling....201
7.11.1. Opioidergic Mechanisms....201
7.11.2. Acute Actions of Opioids....201
7.11.3. Tolerance to Opioids....202
7.11.4. G-protein Biased Opioid Agonists....204
7.11.5. Developing the Two-pathway Signalling Profile....205
7.12. Future Directions....206
7.12.1. Short-Term Goals....206
7.12.2. Medium Term Goals....207
7.12.3. Long-Term Goals....209

7.13. Conclusion.....209

References.....210

Table of Figures

CHAPTER 1 General Introduction..... 1

1.1. Generalised schematic of GPCR activation.....8
1.2. Indication of G-protein dependent and independent signalling from the MOPr.....10
1.3. Schematic of the MOPr and key phosphorylation residues.....12
1.4. Differential development of tolerance to desirable and undesirable effects of heroin.....17
1.5. Comparative blood levels of morphine and ethanol in fatal heroin overdoses.....20

CHAPTER 2 Materials and Methods..... 36

2.1. Typical mouse respiratory trace in air.....41
2.2. Calculation of area under the curve.....46
2.3. Timeline of tail flic experiments with a drug pre-treatment.....49
2.4. Acute tolerance to fentanyl antinociception.....50
2.5. Ethanol diet protocol.....53

CHAPTER 3 Acute Effect of Opioids..... 65

3.1. Dose dependent depression of minute volume by morphine.....67
3.2. Depression of respiratory frequency but not tidal volume by morphine.....69
3.3. Baseline minute volume does not correlate with extent of respiratory depression...69
3.4. Acute morphine antinociception.....70
3.5. Dose dependent depression of minute volume by oxycodone.....71
3.6. Depression of respiratory frequency but not tidal volume by oxycodone.....73
3.7. Acute oxycodone antinociception.....73
3.8. Contribution of KOPr and DOPr to oxycodone respiratory depression.....75
3.9. U69,593 antinociception inhibited by Nor-BNI.....76
3.10. Contribution of KOPr and DOPr to morphine respiratory depression.....77
3.11. Dose dependent depression of minute volume by methadone.....79

3.12. Depression of respiratory frequency but not tidal volume by methadone.....80
3.13. Acute methadone antinociception.....80
3.14. Dose dependent depression of minute volume by fentanyl.....83
3.15. Fentanyl depresses both respiratory frequency and tidal volume.....84
3.16. Antagonism of morphine and fentanyl respiratory depression by naloxone 0.3 mg/kg.....86
3.17. Antagonism of morphine and fentanyl respiratory depression by naloxone 1 mg/kg.....86
3.18. Antagonism of morphine and fentanyl respiratory depression by naloxone 3 mg/kg.....86
3.19. Acute fentanyl antinociception.....89

CHAPTER 4 Development of Tolerance to Opioids.....93

4.1. Induction of morphine tolerance by repeat frequent morphine injections.....96
4.2. Induction of morphine tolerance by twice daily morphine injections.....97
4.3. Induction of morphine tolerance by morphine pump implantation.....99
4.4. Induction of morphine tolerance by morphine pellet implantation.....101
4.5. Induction of morphine tolerance by low oxycodone pump implantation.....104
4.6. Induction of morphine tolerance by medium oxycodone pump implantation.....105
4.7. Induction of morphine tolerance by high oxycodone pump implantation.....106
4.8. Comparison of oxycodone treatments and the induction of tolerance.....107
4.9. Induction of morphine tolerance by methadone pump implantation.....109
4.10. Acute tolerance to fentanyl antinociception.....120

Chapter 5 Mechanisms of Opioid Tolerance.....116

5.1. Effect of tamoxifen on morphine induced tolerance to morphine respiratory depression..... 120
5.2. Effect of calphostin C on morphine induced tolerance to morphine respiratory depression..... 122
5.3. Induction of morphine tolerance in PKCα knock-out mice.....124
5.4. Effect of SP600125 on morphine induced morphine tolerance.....126
5.5. Effect of calphostin C on medium oxycodone induced tolerance to morphine respiratory depression.....128
5.6. Effect of calphostin C on high oxycodone induced tolerance to morphine respiratory depression..... 130
5.7. Effect of calphostin C and tamoxifen on methadone induced tolerance to morphine respiratory depression.....132
5.8. Effect of compound 101 on acute tolerance to fentanyl antinociception.....134
5.9. Effect of compound 101 on acute tolerance to fentanyl respiratory depression.....136
5.10. Effect of calphostin C on acute tolerance to fentanyl antinociception.....138
5.11. Effect of calphostin C on acute tolerance to fentanyl respiratory depression.....139

Chapter 6 Opioid Tolerance and Polydrug Abuse..... 146

6.1. Effect of acute ethanol on morphine induced tolerance to morphine respiratory depression..... 150
6.2. Effect of acute ethanol on medium oxycodone induced tolerance to morphine respiratory depression.....153
6.3. Effect of acute ethanol on high oxycodone induced tolerance to morphine respiratory depression..... 156
6.4. Consumption of prolonged ethanol diet and mouse weight.....159
6.5. Effect of prolonged ethanol diet on the development of morphine tolerance.....161
6.6. Effect of prolonged ethanol diet on the expression of morphine tolerance.....163
6.7. Brain and plasma levels of ethanol and morphine following prolonged ethanol diet and morphine tolerance induction.....165

6.8. Effect of D-penicillamine on ethanol reversal of morphine induced tolerance to morphine respiratory depression.....167
6.9. Effect of acute acetaldehyde on morphine induced tolerance to morphine respiratory depression..... 169
6.10. Effect of acute pregabalin on morphine induced tolerance to morphine respiratory depression..... 172
6.11. Effect of acute pregabalin on medium oxycodone induced tolerance to morphine respiratory depression.....175
6.12. Effect of acute pregabalin on high oxycodone induced tolerance to morphine respiratory depression.....177
6.13. Effect of acute pregabalin on methadone induced tolerance to morphine respiratory depression..... 180

Chapter 7 General Discussion.....186

7.1. Drugs involved in U.S. overdose deaths 1999-2017.....191

Table of Tables

CHAPTER 2 Materials and Methods…….. 36

2.1. Calculation of percent of baseline minute volume.....45

2.2. Composition of control and ethanol diets..... 51

2.3. Stock opioid solutions for calibration of plasma morphine levels.....56

2.4. Calibration curve for plasma morphine levels..... 58

2.5. Standard concentration of ethanol in plasma.....59

2.6. Example of a two by two factorial.....62

Table of Appendices

Appendix 1: Ethanol Reversal of Tolerance to the Respiratory Depressant Effects of Morphine..... 222

Appendix 2: Effect of Tamoxifen and Brain-Penetrant Protein Kinase C and c-Jun N-Terminal Kinase Inhibitors on Tolerance to Opioid-Induced Respiratory Depression in Mice..... 234

Table of Abbreviations

% MPE	Percent maximum possible effect
Arr-3 KO	Arrestin-3 knock out
ATP	Adenosine-triphosphate
AUC	Area under the curve
BAC	Blood Alcohol content
β-ARK 1	β-adrenergic receptor kinase 1 inhibitor
cAMP	cyclic adenosine monophosphate
C101	Compound 101
CC	Calphostin C
CDC	Centre for Disease Control
Ctrl	Control
CYP3A4	Cytochrome P450 3A4
DAMGO	[D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin
DF	Degrees of freedom
DMSO	Dimethyl sulfoxide
DOPr	δ-opioid receptor
DP	D-penicillamine
DPT	Differential Pressure Transducer
DSM-V	Diagnostic and Statistical Manual of Mental Disorders - V
EF	Expiratory Flow
EtOH	Ethanol
F	F - Ratio
GABA	γ-aminobutryic acid receptor
GDP	Guanosine-5'-diphosphate
GIRK	G-protein-coupled inwardly-rectifying potassium channels
GPCR	G-protein coupled receptor
GRK	G-protein receptor kinases
GTP	Guanosine-5'-triphosphate

GTPγS	Guanosine-5'-*O*-(3-[^{35}S]thio)triphosphate
HEK 293	Human endothelial kidney 293 cells
H-oxy	High Oxycodone
IF	Inspiratory Flow
i.p.	intraperitoneal
JNK	c-Jun N-terminal Kinase
KO	Knock out
KOPr	κ-opioid receptor
LC	Locus coeruleus
LTC	L-type calcium channels
M3G	Morphine-3-gluconoride
M6G	Morphine-6-gluconoride
MOPr	μ-opioid receptor
MV	Minute volume
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
Nor-BNI	Nor-Binaltorphimine
OIRD	Opioid induced respiratory depression
ORL-1	Opioid-like-receptor
OST	Opioid substitution therapy
oxo-M	Oxotremerine-M
PEF	Peak expiratory flow
PIF	Peak inspiratory flow
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
preBötc	pre-Bötzinger complex
s.c.	subcutaneous
TMX	Tamoxifen
TV	Tidal Volume
Veh	Vehicle

VSCC	Voltage sensitive calcium channels
WHO	World Health Organisation

1.0 General Introduction

1.1 A Brief History of Opioid Use

The use of opioids has been documented as far back as 3'500 BC in ancient Egypt (Hobbs, 1998), with crude opiate extracts from poppies used as potent analgesics. Over time this led to the development of morphine as the prototypical opioid for acute pain relief (Schug et al., 1992). The American civil war was the first large scale conflict to use morphine on mass as an analgesic in field surgeries. Following the cessation of hostilities between the north and the south, an unintended consequence of liberal morphine use became apparent in the epidemic of morphine addicted soldiers (Lewy, 2014). Opioid addiction was thought to be remedied however, when in 1874, the development of heroin in Germany heralded a supposedly none-addictive opioid (Sneader, 1998). Heroin has since become one of the world's most problematic drugs of abuse.

Every year, thousands of opioid users around the world suffer a fatal opioid overdose, with respiratory depression being the primary cause of death (White and Irvine, 1999). Opioid induced respiratory depression can be mitigated in a controlled therapeutic environment, but the chaotic lives and polydrug habits of opioid addicts lead to a vast increase in the likelihood of opioid overdose occurring.

Addiction to an opioid now comes not just from the use of heroin, but also the spiralling misuse of prescription opioids, often originating from a legitimate need for pain relief from chronic conditions (Okie, 2010). This has led to more opioid addicted individuals than in the past, abusing a greater variety of opioids than in the past.

It has been over 140 years have passed since the development of heroin, yet the need to understand the underlying mechanisms of opioids and the physiological ramifications of opioid use is more pertinent and pressing than ever before.

1.2 Abused Opioids: Heroin, Methadone, Oxycodone and Fentanyl

Heroin, oxycodone, methadone and fentanyl are the key opioids of interest within this thesis. Heroin remains an ever-important drug of interest due to the continued use and abuse of heroin around the world. Methadone is one of the major substitution therapies administered as a means of aiding heroin addicts in decreasing their heroin intake and becoming abstinent to heroin use altogether (Faul et al., 2017, Hickman et al., 2018). Oxycodone has, over the past two decades, become a major prescription drug of abuse in the USA, resulting in accidental overdose deaths during illicit use as well as legitimate therapeutic use (Cicero et al., 2011, Inciardi et al., 2010). However, it is fentanyl, in recent years, that has driven the large increase in the number of opioid related fatalities in the USA (Hedegaard et al., 2017a), with sporadic outbreaks of fentanyl related overdoses in other countries around the world (Pichini et al., 2017). Fentanyl represents an emerging and challenging evolution of the opioid epidemic.

The factors present in the USA have led to what could be described as a 'perfect storm' where prescription opioids have presented the development of an increasing population of opioid dependent or addicted members of society. This has led to an increasing population of opioid users abusing illicit opioids when they can no longer legitimately access prescription opioid. This has led to fluctuating demographics of individual opioid use, as users switch to cheaper and more accessible opioids (Kolodny et al., 2015, Lankenau et al., 2012, Mars et al., 2014).

1.2.1 Heroin

Diacetylmorphine, more commonly known as heroin, is abused in many countries around the world. The supply of heroin is often affected by global events that can mean there are fluctuations in the availability and purity of street heroin (Ciccarone, 2009). Recent trends suggest that both the availability and purity of heroin has been increasing (Mars et al., 2015). Originally, this did not appear to be instigating a rise in heroin overdose deaths, however, recent data suggest that in the USA heroin overdose deaths are now increasing after being relatively stable for many years (Compton et al., 2016, Okie, 2010).

1.2.1.1 Heroin Metabolism and Pharmacology

Heroin itself does not bind to any opioid receptor, instead it functions as a very efficient, and highly lipid soluble pro-drug that penetrates the brain far quicker than its major metabolites would otherwise be able to do (Corbett et al., 2006, De Gregori et al., 2012, Inturrisi et al., 1983). The major metabolite of heroin is 6-monoacetyl-morphine (6MAM) that is swiftly metabolised to morphine (Corbett et al., 2006, De Gregori et al., 2012). Due to the difference in heroin and morphine *in vivo* being primarily the rate of onset, morphine was considered an appropriate agonist to use in place of heroin for this thesis; this was also due in part to the relative availability of morphine over heroin. Both morphine and 6MAM have near selective affinity for the μ-opioid receptor subtype (De Gregori et al., 2012).

Despite the use of morphine as the prototypic analgesic, morphine has relatively low efficacy at the MOPr (Kelly, 2013), something shared by its metabolites. Further metabolism of morphine occurs in the form of glucuronidation, producing both morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) in a 90%-10% ratio respectively (De Gregori et al., 2012). M3G and M6G differ significantly in their affinity for the μ-opioid receptor with M3G having no affinity for MOPr binding compared to a relatively high affinity for MOPr binding shown by M6G, similar to that of morphine (Gottas et al., 2013, Kelly, 2013). M6G also has similar efficacy at the MOPr to morphine, though its rate of clearance is considered to be markedly slower (Gottas et al., 2013). M3G has been suggested to act at other receptors such as the Toll like receptor family, interestingly this is thought to act antagonistically to the actions of morphine and M6G at the MOPr (Lewis et al., 2010).

1.2.2 Methadone

Methadone is one of the most common substitution therapies for heroin addicts. Unlike heroin methadone is administered orally and is thought to provide a means of preventing extreme withdrawal from heroin abstinence as well as providing tolerance to the effects of heroin in the case of relapse, thus helping prevent heroin overdose (Faul et al., 2017, Hickman et al., 2018). However, there is growing evidence that methadone overdose is also a problem among the methadone substitution population (Hickman et al., 2018, Kimber et al., 2015). One additional variable involved in methadone us is the variation in rates of prescription for the modality of pain relief and opioid substitution therapy, with little research defining the difference in response regarding respiratory depression in these divergent fields.

1.2.2.1 Methadone Metabolism and Pharmacology

Methadone has a high binding specificity for the MOPr and so is thought to primarily mediate its actions through MOPr agonism (Whistler et al., 1999). Methadone has a half-life of 15-60 hours in humans and is primarily metabolised peripherally in the liver to two inactive metabolites 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine and 2-ethyl-5-methyl-3,3- diphenyl-1-pyrroline (Grissinger, 2011). The metabolism of methadone occurs through multiple cytochrome P450 enzyme sub-types (Kapur et al., 2011). Methadone metabolism in humans is known to vary considerably, and the plasma level of methadone in patients receiving methadone substitution therapy is not considered a direct correlate of brain levels of methadone levels (Grissinger, 2011, Kapur et al., 2011). This is due to variation in the rate that methadone is transported across the blood brain barrier.

There is evidence within the literature that methadone is a substrate of the cytochrome transporter p-glycoprotein which facilitates transportation across the blood brain barrier (Rodriguez et al., 2004), and so inter-human variation makes methadone highly specific to the individual when calculating the required dosage.

In the mouse, the half-life of methadone is considerably reduced to 2 hours due to an enhanced metabolic rate (Kalvass et al., 2007b). The primary metabolites of methadone remain the same in the mouse, however the enhanced rate of metabolism necessitates a greater dose, than that of morphine, to produce a consistent plasma level of methadone.

1.2.3 Oxycodone

Oxycodone makes up a large percentage of therapeutic opioid prescriptions, particularly in the USA (Kolodny et al., 2015). The USA has seen the greatest rise in oxycodone overdose due to several factors. The prescription culture of the USA has led to a relatively free availability of oxycodone as a pain relief prescription (Elbe et al., 2015). This problem has been severely exasperated by the branding of OxyContin (a patented formulation of oxycodone) as less addictive and less prone to overdose and ensuing fatal respiratory depression (Jayawant and Balkrishnan, 2005). OxyContin has subsequently been demonstrated to be highly addictive and dangerous, though this has led to formulation alterations that do appear to decrease its abuse liability (Coplan et al., 2016).

The problem of oxycodone abuse is still prevalent and important within the USA, however, there has been data to suggest that the population that abuse oxycodone, and oxycodone fatalities has begun to decline (CDC-Wonder, 2017), as users switch to cheaper and more accessible illicit opioids such as fentanyl and heroin (Frank et al., 2015).

1.2.3.1 Oxycodone Metabolism and Pharmacology

Oxycodone has been described as an agonist at all three major opioid receptor subtypes (MOPr, DOPr and KOPr) (Ordonez Gallego et al., 2007), though in general oxycodone is thought have relative selectivity for the MOPr. Whether agonist activity at multiple opioid receptor subtypes is important in the abuse liability of oxycodone is not currently known.

Oxycodone has a half-life of 3-4 hours in humans (Ordonez Gallego et al., 2007), which is significantly diminished in mice due to enhanced metabolism (Raehal and Bohn, 2011). Oxycodone undergoes a single phase 1 metabolism through a single known cytochrome P450 enzyme (CYP3A4) (Ordonez Gallego et al., 2007, Klimas et al., 2013). This is known to produce a small fraction of oxymorphone as an active metabolite. The impact of oxymorphone as a percentage of the overall response to oxycodone is thought to me minimal (Klimas et al., 2013). However, there is evidence to suggest that benzodiazepines can interfere with oxycodone metabolism producing a prolonged concentration of oxycodone in comparison to its normal half-life (Fields et al., 2015), this would make users of oxycodone and benzodiazepines at greater risk of overdose.

1.2.4 Fentanyl

Fentanyl has previously been problematic as a short-lived epidemic in the USA, with a significant increase in fentanyl seizures and fentanyl related overdose deaths occurring in 2008 (CDC, 2015). However, in the years since 2015, fentanyl supply and use within the USA has reached unprecedented levels with fentanyl related overdose deaths superseding the number of overdose deaths recorded from heroin and prescription opioids (Hedegaard et al., 2017a, CDC, 2015).

The higher potency of fentanyl has allowed relatively easy trafficking of small fentanyl packages through legitimate national and international postage systems, which are then subsequently cut to a lower purity, or used to enhance the "quality" of heroin (Ciccarone, 2009, Dasgupta et al., 2013).

However, miniscule errors in the mixture of fentanyl and heroin by dealers or in the preparation of doses by addicts can result in swift overdose fatalities, due to the far greater potency of fentanyl. Unlike the previous epidemic involving fentanyl, there does not appear to be any indication that the current epidemic will end swiftly, and so a thorough re-assessment of how fentanyl induces overdose as well as how these overdose cases are handled is necessary to prevent loss of life as much as possible.

1.2.4.1 Fentanyl Metabolism and Pharmacology

Fentanyl was the first synthetic MOPr specific agonist to be developed as part of the now broad family of MOPr agonists the "fentanils" (Stanley, 2014). Fentanyl is an extremely potent opioid agonist, approximately 100-fold more potent than morphine at inducing both analgesia and respiratory depression in humans. However, it has a relatively short half-life of 1-2 hours when administered intravenously (Stanley, 2014), compared to 3-6 hours for morphine (Hasselstrom and Sawe, 1993).

Fentanyl is primarily metabolised through a cytochrome P450 enzyme (CYP3A4) in the liver (Feierman and Lasker, 1996). The major metabolite of fentanyl, norfentanyl, is inactive at the opioid receptors (Vardanyan and Hruby, 2014). However, the single enzyme isoform that metabolises fentanyl, is, like oxycodone, also the primary enzyme for several benzodiazepines. As such, though there has not been direct experimental examination of this possibility, it has been suggested that co-administration of benzodiazepines and fentanyl will result in competition for CYP3A4 binding and thus enhance the effect of fentanyl on the CNS (Fields et al., 2015).

1.3 Opioid Receptor Activation

The opioid receptor family contain three major sub-types; the μ-opioid receptor (MOPr), the δ-opioid receptor (DOPr), the κ-opioid receptor (KOPr) as well as the minor sub-type nociception receptor (NOPr). Opioid receptors are G-protein coupled receptors (GPCRs) and as such contain the stereotypical seven transmembrane domains that characterise GPCRs. G-proteins themselves vary in structure and as such not all GPCRs bind to the same G-protein sub-units. Opioid receptors primarily bind to $G_{i/0}$ G-proteins, with the primary points of interaction being the C-terminus tail of the receptor as well as the third intracellular loop of the receptor. G-proteins typically consist of three separate sub-units, the α-subunit, the γ-subunit and the β-subunit; however, the latter two subunits work in a β/γ-subunit complex for the purpose of signalling (Williams et al., 2013).

The binding of an opioid ligand at the extracellular domain of the receptor is thought to result in a conformational change in the structure of the opioid receptor (Kelly et al., 2008, Kenakin, 2003). This conformational change is not homologous across all opioid ligands or even opioid agonists versus opioid antagonists. The difference in conformational change, caused by differential binding of opioid ligands within the orthosteric binding site of an opioid receptor is widely believed to be a key factor in the differential downstream signalling cascades seen between receptors (Kelly et al., 2008, Kenakin, 2003). Broadly speaking however, the conformational change allows the activation of G-proteins to occur or the recruitment of other intracellular signalling messengers such as arrestin or G-protein regulating kinase (GRK).

Typical agonist activity at an opioid receptor and the activation of the G-protein signalling pathway is depicted in Figure 1.1. The binding of an opioid agonist (e.g. morphine) causes a conformational change in the opioid receptor that allows the exchange of guanosine-5'-diphosphate (GDP) for guanosine-5'-triphosphate (GTP) on the G-protein's α-subunit, allowing the α-subunit to then dissociates from the receptor. Simultaneously the α-subunit dissociates from the β/γ-subunit. The dissociation of the α-subunit allows both the α-subunit and the β/γ-subunit to engage with intracellular second messaging systems as well as activate other membrane bound proteins and ion channels.

The dissociated α-subunit inhibits adenylyl cyclase which leads to a decrease in cyclic adenosine monophosphate (cAMP) production, simultaneously the β/γ-subunit complex activates G-protein-coupled inwardly-rectifying potassium (GIRK) channels (Bailey et al., 2009a, Bailey et al., 2009b, Ikeda et al., 2000) The activation of GIRK channels causes an efflux of potassium ions (K^+) from the intracellular space. The net efflux of positively charged K^+ ions lead to the hyperpolarisation of the cell. This occurs in conjunction with β/γ-subunit dependent inhibition of N-type voltage sensitive calcium channels (VSCC), leading to a decrease in calcium ion influx (Soldo and Moises, 1998).

The net effect of morphine binding to the MOPr and the subsequent activity of the G-protein α-subunit and β/γ-subunit complex is to generate an intracellular condition that is not conducive to signalling as the excitability of the cell is decreased through both hyperpolarisation and a decrease in cAMP.

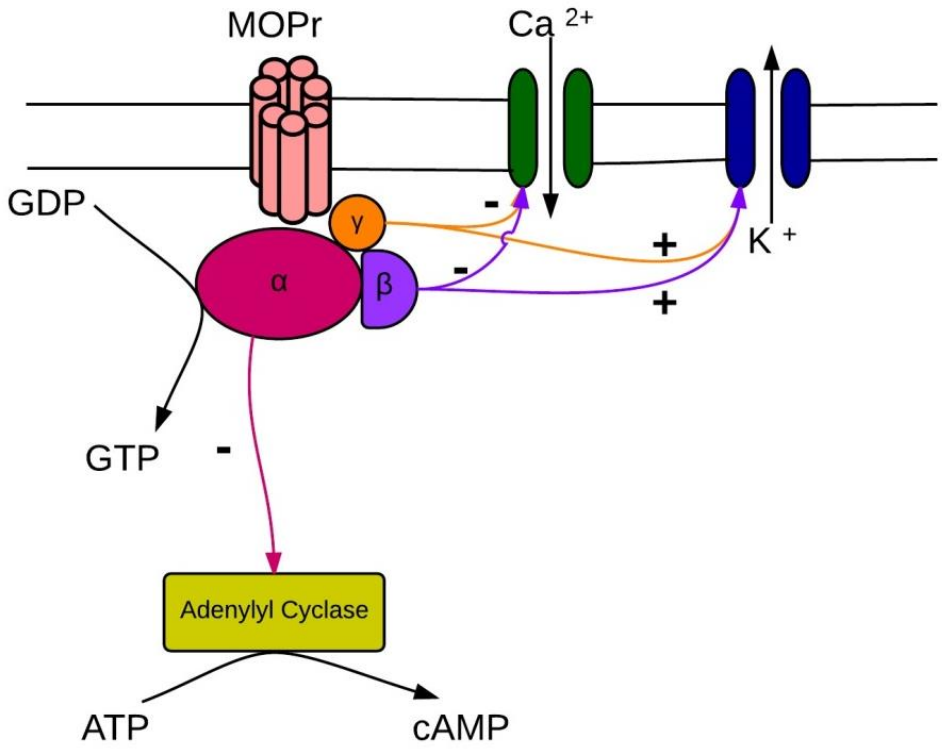


Figure 1.1. Generalised schematic of GPCR activation. *The binding of an agonist to the MOPR results in decreased calcium (Ca^{2+}) influx and increased potassium (K^+) efflux by Gβ/γ, and a decrease in cAMP production by Gα that causes an overall decrease in neuronal excitability and signal transmission.*

1.4 μ - Opioid Receptor Location

The opioid agonists used within this thesis are generally considered to either bind exclusively to the MOPr or conduct the majority of their agonist activity through the MOPr. With this in mind, it is only the location and molecular biology of the MOPr that will be considered for this introduction.

MOPrs are distributed widely throughout the brain, spinal cord and gastrointestinal tract. The MOPr in particular is thought to be a key receptor in signalling through the mesolimbic dopaminergic reward pathway. This reward pathway is considered to form a fundamental aspect with regard to the development of addiction (Trigo et al., 2010). Both the ventral tegmental area and the nucleus accumbens, and MOPr activity there within, are frequently investigated to ascertain the importance of opioids in addiction (Martin et al., 2008).

The spinal dorsal horn, brain stem, thalamus and cortex are key areas of ascending pain pathways and the periaqueductal grey, rostral ventral medulla and nucleus raphe magnus are key areas of descending pain pathways (Inturrisi, 2002). These all express the MOPr to varying degrees and are important functional areas in the pain-relieving effects of opioids.

1.5 μ - Opioid Receptor Desensitization and its Relation to Tolerance

Opioid agonist activation of the MOPr has been shown to recruit a large variety of intracellular proteins (Williams et al., 2013). Recruited proteins can bind directly with the MOPr or be activated by the dissociating G-proteins from the MOPr, though G-protein activation is it not necessary for intracellular signalling to occur. Variably, recruited proteins play important roles in receptor desensitization, internalisation, recycling, and re-insertion of the receptor (Williams et al., 2013). Figure 1.2, schematically highlights the large variety of proteins and second messenger systems that can be activated by opioid agonist binding to the MOPr.

There is a substantial body of literature that has investigated the propensity of different opioid agonists to desensitise the MOPr. Desensitization represents a decrease in the signalling capacity of receptors in the short term i.e. <10 minutes. Different opioid agonists have been shown to produce varying degrees of MOPr desensitization. This has been linked to inherent efficacy of opioid ligands, the dwell time of opioid ligands in both orthosteric and allosteric binding sites, as well as the bias activation of different intracellular signalling pathways. Bias activation has in particular focused on G-protein signalling versus arrestin recruitment to the MOPr.

As well as the scientific investigation of desensitization itself, there has been a continued examination of how desensitization of the MOPr relates to the observed phenomenon of opioid tolerance. Tolerance here is defined as an adaptation in which at least one effect of a drug (in this case opioid agonists) is reduced following exposure to a drug over time (Johnson et al., 2006, Levitt and Williams, 2012). While both desensitization and tolerance represent a loss of function over time, they do not necessarily represent a cause and effect pathway in which desensitization inevitably leads to tolerance. As such there has been a continued movement of research seeking to define a connection between these desensitization and tolerance, or indeed to delineate these two occurrences (Kelly, 2013).

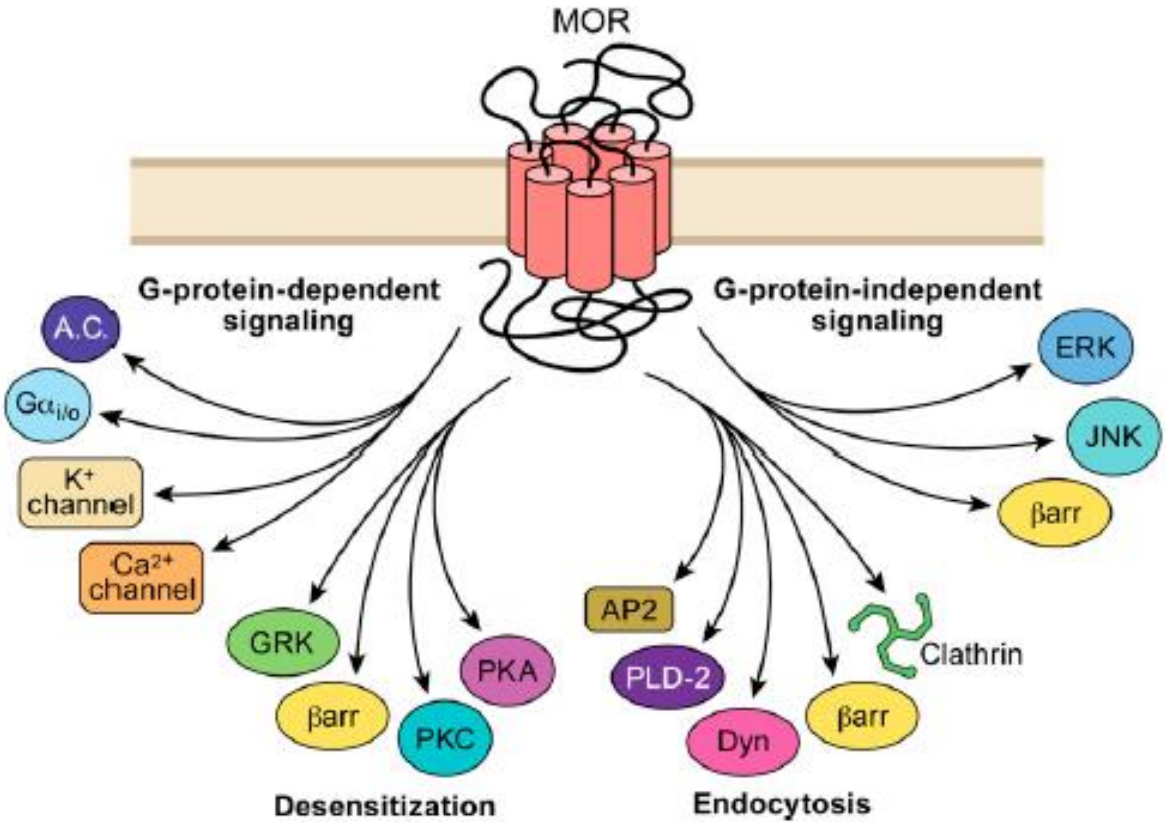


Figure 1.2. Indication of G-protein dependent and independent signalling from the MOPr as well as proteins thought to be activated in both desensitization of the receptor and its internalisation via endocytosis*. Adapted from Williams et al, (2013).*

1.6 Phosphorylation of the MOPr by GRK and PKC

Phosphorylation of the MOPr is considered to be a major component of MOPr desensitization (Kelly et al., 2008) (Figure 1.3). Both protein kinase C (PKC) (as a function of G-protein signalling) and G-protein receptor kinases (GRKs) are considered fundamentally important kinases in the phosphorylation of the MOPr leading to MOPr desensitization. However the recruitment of these kinases has been shown to be largely agonist dependent (Bailey et al., 2006) with different MOPr agonists shown to selectively bias the recruitment of different kinases to enable receptor desensitization. Johnson *et al*, (2006) demonstrated that in human endothelial kidney 293 (HEK 293) cells expressing both GIRK channels and MOPr the application of MOPr agonists [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin (DAMGO) and morphine caused robust desensitization. Expression of a dominant negative GRK2 mutant in the HEK cells largely prevented DAMGO desensitization of the MOPr but left morphine desensitization largely intact. Conversely, the application of a PKC inhibitor largely prevented morphine desensitization but largely left DAMGO desensitization intact. This demonstrated that kinase dependent desensitization of the receptor was specific to the MOPr agonist.

Further evidence to support the bias of DAMGO to recruit a GRK dependent method of MOPr desensitization compared to Morphine recruitment of PKC was demonstrated in locus coeruleus (LC) neurones (Bailey et al., 2009b). Application of a PKCα inhibitor prevented morphine desensitization of the MOPr but left DAMGO desensitization intact. Other PKC isoform inhibitors were not able to inhibit morphine dependent desensitization and LC neurones from PKCα knock-out mice were also unable to express morphine desensitization. Over expression of a GRK2 dominant negative mutant (but not GRK6) once again prevented DAMGO desensitization yet left morphine desensitization intact.

These investigations demonstrated that opioid agonists at the MOPr have the propensity to show bias to the cellular mechanism recruited to enable MOPr phosphorylation and subsequent desensitization. This has since been shown to occur for a large number of MOPr agonist that range from being neutral in their recruitment of cellular kinases to expressing varying degrees of bias to recruit different isoforms of GRK and PKC (Kelly, 2013). These actions may form a crucially important component of how tolerance develops *in vivo* differentially to different MOPr opioid agonists.

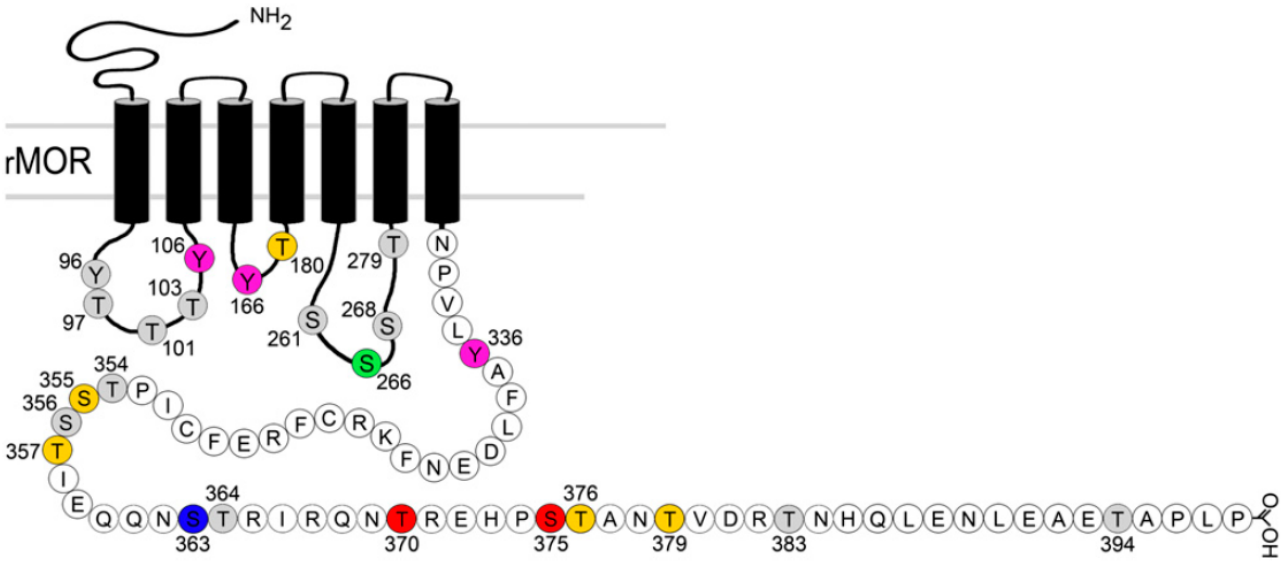


Figure 1.3. Schematic of the MOPr along with an expanded version of key amino acid sequences in MOPr intracellular loops and the c-terminus*. Shaded/coloured amino acids represent residues that have strong evidence for being key phosphorylation site that may selectively activate cellular pathways/proteins dependent on ligand bias agonism. Adapted from Williams et al, 2013.*

1.7 Desensitization and Internalisation of the MOPR

Desensitization and internalisation occur over a relatively short time frame (Williams et al., 2013) and are both thought to be functionally significant components in the development or lack of development of tolerance to the effect of opioids *in vivo*.

MOPr opioid agonist induced desensitization is often discussed as the precursor to tolerance at the MOPr, and there is certainly evidence to support the necessity of desensitization not only in the development of tolerance but also the maintenance of tolerance (Bailey et al., 2009a). However other investigations have indicated that opioid desensitization and opioid tolerance exist as mutually exclusive components of an overall decreased response to opioids over time which represents 'tolerance' as a whole (Levitt and Williams, 2012). Furthermore some investigations have suggested that tolerance to opioids, specifically morphine, is developed more readily due to a relative inability to cause desensitization and subsequent MOPr recycling (Whistler, 2012).

Bailey *et al*., (2009a) demonstrated that cellular tolerance generated *in vivo* through a 3-day subcutaneous 200mg/kg slow release morphine formula was underpinned by profound MOPr desensitization in LC neurones maintained in 1μM morphine solution to prevent withdrawal. Washout of the morphine solution for 2-4 hours resulted in reversal of MOPr desensitization and therefore tolerance suggesting MOPr desensitization by morphine is required for tolerance to the effects of morphine.

Levitt and Williams, (2012) treated rats with 50 mg/kg/day morphine for 6 or 7 days (using osmotic mini-pumps) before measuring the morphine response in LC neurons in both the presence and absence of a 1μM morphine solution. LC neurons from treated rats maintained in artificial cerebral spinal fluid showed a significantly decreased response to morphine compared to naïve controls, with this response defined as long lasting cellular tolerance. The morphine response was further decreased in 1 μM morphine solution-maintained LC neurons. Levitt and Williams defined this additional decrease as desensitization 'on top' of tolerance, concluding that tolerance and desensitization were distinct but worked cumulatively to decrease the morphine response in LC neurons.

Discrepancies between these two investigations may be attributable to length of prolonged morphine treatment as well as the size and manner of dosing. However, both investigations clearly highlight the significance of desensitization to the overall decrease in morphine response which is not due to a decrease in receptor expression as neither investigation found a shift in the EC_{50} but instead saw a decrease in the maximum response to morphine as would be expected for a partial agonist.

1.8 Respiratory Control and Opioids

The physiological drive for respiration has multiple points of control. The midbrain contains a pontine-medullary complex formed of several nuclei that are thought to be key in the control of inspiratory-expiratory rhythm (Lalley et al., 2014a). Key nuclei in this area include the ventral respiratory column, the Kölliker-fuse complex and the Bötzinger as well as pre-Bötzinger complex (preBötc) areas. The preBötc has been noted as potentially forming the dominant rhythm generating nucleus within the pontine-medullary complex. Of importance is that all of these nuclei contain MOPrs, indeed application of MOPr agonists by discreet infusion into the preBötc have been shown to decrease neuronal activity and produce marked respiratory depression (Qi et al., 2017). This does not exclude the important or necessity of other nuclei in respiratory rhythm generation; indeed, it has been argued that the preBötc is in fact none-essential to rhythm generation (Lalley et al., 2014a).

Respiration depends heavily on a sensitive feedback loop that allows the body to respond to current physiological parameters that necessitate changes in breathing to maintain a homeostatic balance. These are primarily mediated by chemosensitive and mechanosensitive receptors. Both central and peripheral chemosensory bodies detect a rise in blood CO_2 concentration by proxy, due to the decreased blood pH caused by an increased amount of solubilised CO_2 (LeGrand et al., 2003). The preBötc, nucleus tractus solitarus (NTS) and raphe nucleus contain central chemoreceptors and these areas have also been shown to display sensitivity to MOPr agonists, with agonist application decreasing neuronal activity and output in these areas (Pattinson, 2008, Lalley et al., 2014a).

Peripheral receptors are found in the aortic arch and carotid bodies. Given that the carotid body chemoreceptors have direct connections to the NTS, they are considered the more important of the two peripheral chemosensory areas. The direct innervation of the carotid bodies from the NTS allows swift modulation of respiratory rate in response to a hypercapnic environment (Pattinson, 2008). The aortic arch still contributes to the respiratory response seen following hypercapnic conditions, but to a much lesser extent than the carotid bodies. However, the role of MOPrs and opioid agonist administration on peripheral chemosensory activity remains to be fully elucidated.

Other than peripheral chemoreceptors there are also peripheral mechanoreceptors. These peripheral mechanoreceptors act as pulmonary stretch receptors in the lung with the role of preventing over-inflation. Additionally, if tidal volume decreases, indicating a decrease in lung inflation, they aid in the mediation of an increased respiratory rate to compensate (LeGrand et al., 2003). There is some literature to indicate they may be affected directly my MOPr agonist administration, but the overall impact of these mechanosensitive pulmonary receptors on respiratory depression is thought to be minimal (Chow and Read, 1984).

There is a considerably debate over the prominence of individual nuclei in the hierarchy of respiratory rhythm generation and control. The multitudinous nuclei that have been implicated in respiratory rhythm control indicate that respiratory rhythm is likely to be controlled by a multifaceted interplay between many nuclei (Lalley et al., 2014a, Lalley et al., 2014b). However, MOPrs have been noted at many key nuclei that are known to be important in respiratory rhythm generation, as well as MOPrs being present in chemosensitive areas of respiratory control. Discreet MOPr agonist application into select nuclei and chemosensitive bodies decreases neuronal excitability with the net result of decreased neurotransmitter release. This will inevitably decrease the activity of respiratory controlling nuclei and contribute significantly to opioid induced respiratory depression as well as decrease the sensitivity to feedback loops generated by the enhanced hypercapnic environment. Cumulatively this represents the opioid induced response of respiratory depression.

1.9 Tolerance to Respiratory Depression

The primary cause of death in instances of fatal opioid overdose is respiratory depression (White and Irvine, 1999). Respiratory depression is the most severe side effect of opioid use and misuse. Other side effects include (but are not limited to) nausea, constipation, and sensitisation to nociceptive inputs (Collett, 1998, Foley, 2003, McQuay, 1999). The primary desired effects of opioids in humans are analgesia, anxiolysis and cough suppression in therapeutic use, and euphoria in illicit use. There is mixed evidence within the literature as to whether tolerance develops to all opioidergic effects. Tolerance to opioid induced nausea is reported to be swift, whereas tolerance to opioid induced constipation appears to be almost completely absent (Arner et al., 1988, Brescia et al., 1992, Rowbotham et al., 2003).

In the context of illicit opioid use, the two primary opioidergic effects of concern are euphoria and respiratory depression. Dose escalation of opioids in order to maintain the same level of euphoria is well noted in the addicted populace (Frank et al., 2015). As noted earlier, opioid induced respiratory depression is the primary cause of death in opioid overdose. As such the relative rate and extent of tolerance development to respiratory depression in comparison to euphoria is of great scientific interest and sociological benefit.

There has been relatively little evidence within the scientific literature that tolerance developed to opioid induced respiratory depression. Evidence suggesting that tolerance is absent was presented by Ling et al (1989), in which 8 hour intravenous morphine infusions in rats failing to produce tolerance to respiratory depression whilst antinociceptive tolerance was rapidly acquired (Ling et al., 1989). A more recent publication submitted that intermittent intramuscular injections of heroin (0.5 mg/kg/day) or morphine (16 mg/kg/day) over a 3 day period failed to cause tolerance to opiate respiratory depression in rhesus monkeys breathing 5% CO_2 (Kishioka et al., 2000).

There is some evidence that tolerance to respiratory depression occurs, though requiring a longer period of opioid administration. Repeated administration of morphine (2.5 mg/kg subcutaneous) each day for 10 days was shown to result in significantly attenuated respiratory depression in response to acute morphine (2.5 mg/kg) (Mohammed et al., 2013). However, this publication largely demonstrated an attenuation of a rise in respiratory rate seen following administration of vehicle.

However, Hill et al, (2016), published as part of this investigators previous MSc (Res) (See Appendix 1) demonstrated that tolerance to acute morphine respiratory depression occurred following prolonged morphine treatment with a 75mg morphine pellet implanted subcutaneously in mice for a period of 6 days. The morphine experiments within this publication were entirely the work of the this author (R.Hill).

In addition to pre-clinical scientific evidence of tolerance to opioid respiratory depression, there has been at least one clinical observation of rapid tolerance developing to opioid induced respiratory depression (Inturrisi, 2002). The difference in the reported occurrence of tolerance and indeed rate of onset may be due to species difference, dosing methods, opioid rotation regimes or other factors. However, this discrepancy is an important factor to consider in translation of opioid tolerance research.

1.9.1 Convergence of Tolerance to Opioid Respiratory Depression and Euphoria

As previously stated, the relative occurrence of tolerance to different opioidergic effects, as well as the rate of tolerance onset is highly debated. A seminal review in the field of opioid overdose and polydrug abuse by White and Irvine, (1999); hypothesised an increased risk of lethal overdose in experienced heroin users. The underlying mechanism is hypothesised to be a differential development of tolerance to the euphoric and respiratory depressive effects of heroin.

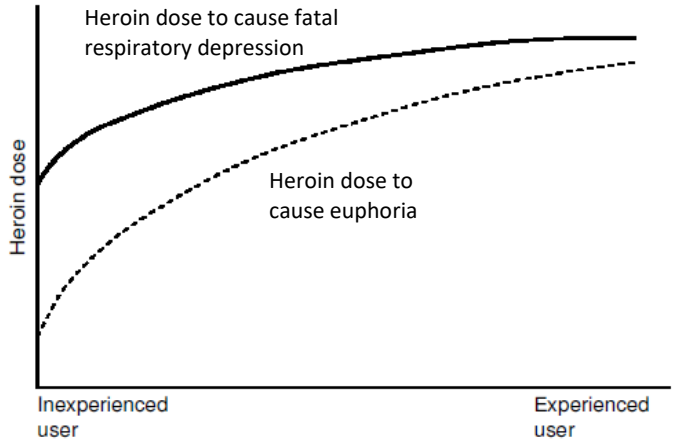


Figure 1.4. Differential development of tolerance to desirable (i.e. euphoria) and undesirable (i.e. respiratory depression) effects of heroin. *Hypothetical model of differential tolerance to euphoria and lethal dose by respiratory depression. Adapted from White and Irvine (1999).*

Figure 1.4 illustrates two hypothetical curves. The solid line represents the required dose of heroin to achieve lethal respiratory depression, compared to the dashed line that illustrates the dose of heroin required to achieve euphoria. Due to the differential development of tolerance to heroin induced euphoria and respiratory depression, as the heroin user becomes more experienced the "safety gap" between a dose of heroin that produces the desired euphoria and undesired lethal respiratory depression becomes narrowed.

Whilst the work of White and Irvine is hypothetical, subsequent etiological evidence supports this interpretation. Data collated in a review by Warner-Smith *et al*, 2001 highlights that the average age of overdosing addicts in Australia is approximately 30 years of age, and lethal overdose occurs primarily in experienced, rather than naïve, heroin users. Whilst dose escalation is known to occur in order to maintain the desired euphoric effect of heroin, it is counter-intuitive that experienced heroin users would overdose more often. Therefore this strongly suggests that there is an effect of prolonged heroin use on tolerance to heroin respiratory depression (Hall and Darke, 1998).

Additional data that substantiates an important discrepancy between tolerances to heroin induced euphoria and respiratory depression is demonstrated in post-mortem toxicology results from fatal heroin overdose victims. Whilst one might expect a heroin overdose victim to have substantially higher levels of heroin and its metabolites present in the blood stream, this is not the case. Post-mortem reports have several times highlighted that the plasma levels of heroin and its metabolites are lower in fatal overdose than the plasma levels of living users (Fig. 1.5) (Monforte, 1977, Darke et al., 2002a, Brewer, 2002).

1.10 Ethanol and Opioids

1.10.1 Aetiological Evidence of Ethanol and Opioids

Heroin users are notoriously poly-drug abusers, with scientific reports illustrating the concurrent abuse of cocaine, benzodiazepines, amphetamines and alcohol (in the form of ethanol) in the heroin addicted population (Darke, 2003). Ethanol is the most prevalent additional drug of abuse seen in the heroin using population (Darke, 2003, Hickman et al., 2008).

Additionally, as well as post-mortem analyses of heroin overdose victims revealing lower than expected levels of plasma heroin, they also reveal an inverse relationship between heroin and blood ethanol content (Ruttenber et al., 1990, Darke and Hall, 1995). The concomitant use of heroin and ethanol has long been regarded as particularly dangerous. However, the canonical understanding of this danger has been interpreted as a summation of both acute heroin respiratory depression, coupled with respiratory depression induced by ethanol (Hickman et al., 2008b).

Indeed, ethanol-induced impairment of judgment may also result in incorrect dosing with heroin, thus leading to a heroin overdose situation on top of ethanol intoxication due to cognitive inhibition of the opioid user (Hickman et al., 2008b). However, there is a notable proportion of overdose death involving ethanol and heroin that have neither high blood concentration of ethanol nor morphine (Fig. 1.5) which suggests that neither summation of respiratory depression by ethanol and heroin has occurred and nor has overdosing of heroin due to ethanol inhibition of cognition (Ruttenber et al., 1990, Darke and Hall, 1995).

This suggests that enhanced respiratory depression occurring from concomitant use of ethanol and heroin may come in the form of pharmacokinetic and pharmacodynamics interactions, or indeed a more nuanced interaction at the level of the MOPr (Hickman et al., 2008) as well as intracellular second messenger systems may be occurring (Darke and Hall, 2003).

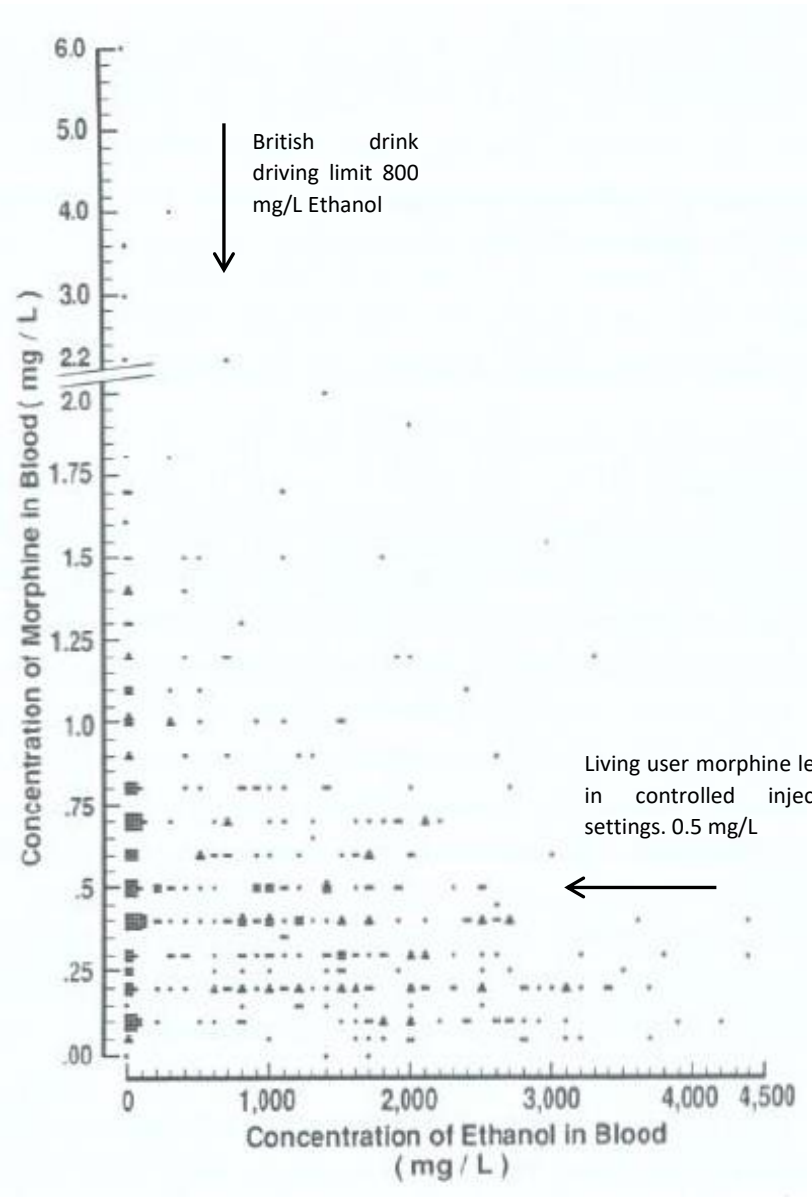


Figure 1.5 Comparative blood levels of morphine and ethanol in fatal heroin overdoses. *A scatter plot of post-mortem morphine blood concentration against ethanol blood concentration showing an inverse correlation, with a large clustering of fatalities at low morphine and high ethanol levels. Adapted from Ruttenber et al; (1990). Living user data from (Brewer, 2002).*

1.10.2 Social Context and Opioid Overdose

As well as considering the polydrug situation of an opioid users, one must also consider the social situation and context of opioid use. Due to the criminalisation of heroin and other illicit opioids, users frequently find themselves incarcerated either due to the use of an opioid itself, or associated crimes committed due to addiction. The forced abstinence of incarceration may cause an underlying decay of tolerance to heroin induced respiratory depression. The rate of opioid overdose in forcibly abstinent addicts die to incarceration has been shown to significantly increase in the weeks following release (Binswanger et al., 2013).

Additionally, there is evidence to suggest that opioid users undergoing methadone maintenance treatment are particularly susceptible to an overdose incidence in the first few weeks so substitution treatment as well as in the first few weeks following cessation of substitution treatment (Hickman et al., 2018, Kimber et al., 2015). These periods of increased risk may reflect a relatively low level of cross tolerance and a decay in tolerance respectively.

Finally, during these periods of decreased tolerance after enforced abstinence or substitution treatment the decay in tolerance to opioid induced respiratory depression may then be obfuscated by the consumption of ethanol during a relapse into opioid use (Hickman et al., 2008b).

1.10.3 Opioid Induced Desensitization and Ethanol in Brain Slices

The effect of ethanol on opioid induced desensitization at the level of the MOPr has previously been investigated in locus coeruleus (LC) slices from rat brains (Llorente et al., 2013). In LC slices, in vitro tolerance was induced through prolonged exposure to opioid agonists (up to 9hrs), of either 1µM morphine or 100nM DAMGO. Following prolonged opioid agonist application Llorente et al (2013), performed both patch-clamp and whole cell electrophysiology recordings in order to measure GIRK channel conductance following acute application of a maximally effective concentration of morphine (30µM).

Prolonged exposure to either morphine or DAMGO significantly reduced GIRK channel conductance following acute morphine application, demonstrating desensitization at the level of the MOPr. This was not due to intrinsic alteration in the GIRK channel, as the peak response to noradrenaline (NA) in both control slices and prolonged opioid slices were unchanged. NA binds to adrenergic receptors that also couple to GIRK channels, so any change in GIRK channel conductance induced by prolonged exposure to either opioid would have also affected the peak current following NA application.

In vivo tolerance was also induced in rats through sub-cutaneous (sc) injection of 200 mg/kg morphine base contained within a slow release formulation. Three days after administration of the slow release formula, rats were killed, and LC brain slices prepared. GIRK channel conductance in response to morphine application was significantly reduced in these LC slices. This again indicated significant desensitization at the level of the MOPr. NA application induced changes in GRIK current remained unaffected.

In LC brain slices prepared from rats rendered tolerant to morphine in vivo, and in LC brain slices that received prolonged morphine in vitro, application of ethanol (20mM) for a period of 10 minutes returned slice sensitivity to acute application of morphine. This appeared to demonstrate a cellular reversal of morphine tolerance at the MOPr, induced by ethanol application. In LC brain slices rendered tolerant to morphine by prolonged DAMGO administration, ethanol application did not reverse cellular tolerance.

Llorente et al (2013), performed additional experiments in human endothelial kidney 293 (HEK293) cells to examine potential mechanisms of ethanol at the level of the MOPr expressed in the HEK293 cells. Radioligand displacement was performed with tritiated naloxone to assess the affinity for morphine binding to the MOPr in the presence and absence of ethanol. Ethanol did not show any alteration in morphine affinity for MOPr binding. Similarly ethanol was shown to have no effect on morphine agonist efficacy, measure through morphine dependent stimulation of guanosine 5'-O-(3-[^{35}S]thio)triphosphate binding membranes prepared from HEK293 cells stably expressing HA-tagged MOPrs.

However, a 15min application of ethanol (20mM) did significantly reduce the level of MOPr phosphorylation induced by a 10min treatment of morphine (30µM). DAMGO induced phosphorylation of the MOPr was unaffected by ethanol.

Llorente et al (2013), demonstrated that ethanol administration was able to reverse cellular tolerance to morphine whether that was induced through *in vivo* or *in vitro* means. This mechanism operated independently of changes in GIRK channel conductance, as well as morphine affinity or efficacy at the MOPr. Additionally, the effect of ethanol was ligand dependent, as ethanol application had no effect on DAMGO induced tolerance.

These experiments indicate that discreet desensitization of the MOPr by morphine but not that by DAMGO can be reversed by ethanol, thereby demonstrating ethanol has the capacity to either resensitize the MOPr or increase morphine efficacy at the MOPr.

1.10.4 Morphine Antinociception in Mice and Ethanol

Further evidence supporting a physiologically significant interaction between opioid tolerance and ethanol has since been demonstrated using in vivo behavioural paradigms in mice (Hull et al., 2013, Hill et al., 2016). Following the sc implantation of a 75mg morphine pellet on the dorsal flank of mice for three days, Hull et al, demonstrated a significant rightward shift of the dose response curve to morphine antinociception in these mice. Antinociception was assessed using the warm water tail flick technique. Similarly, Hill et al (2016), showed that implantation of a 75mg morphine pellet sc in mice for 6 days completely abolished the antinociceptive response to acute morphine (10 mg/kg) on day 6 compared to controls in the warm water tail flick assay.

Both Hull et al (2013), and Hill et al (2016), found that administering an ethanol (0.1-1 g/kg) dose dependently reversed morphine tolerance. However, there are difference in the timing of ethanol administration with Hull et al (2013), administering ethanol 30 minutes prior to the morphine challenge, compared to Hill et al. where ethanol and the morphine challenge were administered concomitantly. Hill et al (2016), also demonstrated maximal reversal of antinociceptive tolerance at a dose of ethanol 0.3 g/kg compared to the dose of 1 g/kg dose of ethanol in Hull et al. This discrepancy may well reflect the difference in time of ethanol administration, reflecting metabolism of ethanol.

However, the fact remains that both investigations found that prolonged ethanol produced profound tolerance to morphine antinociception and administration ethanol was able to return sensitivity to morphine antinociception. This could be termed as a reversal of in vivo tolerance. Equally, in both investigations, ethanol was found to produce no antinociceptive or pronociceptive effect alone.

1.10.5 Morphine Respiratory Depression in Mice and Ethanol

Whilst it appears that ethanol is able to return morphine sensitivity in mice that have been rendered tolerant to morphine antinociception, this does not necessarily apply across all effects of morphine or indeed all opioids. The effect of ethanol on morphine antinociception may be specific to nociceptive neurons, or even spinal reflexes given that both Hull et al (2013), and Hill et al (2016), utilised the tail flick assay (a spinal reflex pathway).

Additionally, regarding the social and aetiological evidence that surrounds the ethanol-opioid interaction, the opioidergic effect of greatest importance is that of opioid induced respiratory depression. As previously discussed, there has been conflicting evidence surrounding the development of tolerance to opioid induced respiratory depression.

The current investigators previous work (Hill et al., 2016) (Appendix 1) demonstrated that tolerance to acute morphine respiratory depression occurred following prolonged morphine treatment with a 75mg morphine pellet implanted subcutaneously in mice for a period of 6 days. Mouse respiration in these experiments was measured using whole body plethysmography whilst chambers were supplied with 5% CO_2 in air to provide a mild, none anxiogenic, hypercapnic environment. This created sufficient respiratory stimulus to provide a very reproducible respiratory paradigm.

Mice administered morphine (10 mg/kg) acutely displayed significant (~40%) respiratory depression. Following 6-day treatment with a sc 75mg morphine pellet, the same dose of acute morphine caused no significant respiratory depression, demonstrating tolerance to morphine respiratory depression.

However, concomitant administration of ethanol (0.3 g/kg) was able to restore morphine respiratory depression in tolerant mice. This was not due to an increase in available morphine within the blood or brain as both were unchanged following ethanol administration.

Additionally, mice that had received acute injections of methadone and buprenorphine followed by implantation of osmotic mini-pumps containing enough drug solution of methadone (50 mg/kg/day) or buprenorphine (5 mg/kg/day) for 6 days were also tolerant to the acute respiratory depressant effects of morphine. Unlike with morphine induced tolerance to morphine respiratory depression, the concomitant administration of ethanol did not reverse tolerance induced by methadone or buprenorphine.

This data correlates with previous investigations (Llorente et al., 2013) that suggest that the ability of ethanol to reverse opioid tolerance is dependent on the opioid ligand administered to induce tolerance. This may reflect the growing understanding that different ligands differentially activate different cellular mechanisms and as such tolerance does develop in a homogenous manner across all ligands (McPherson et al., 2010).

1.11 Alcohol in Society

Alcohol is an extremely available substance worldwide and is socially and morally accepted in many (though not all) cultures. Due to the naturally occurring fermentation process, it has been present in the history of mankind for millennia. It is, however, susceptible to misuse like any drugs of abuse. The burden of none-addicted alcohol use, through the metrics of health, crime and social strife is abundant. Further to this burden are those individuals who are addicted to alcohol alone and also those who abuse alcohol as part of a polydrug arsenal of intoxication. Opioid addicts most commonly also abuse alcohol additionally (Darke, 2003).

Alcohol is primarily consumed by humans for its intoxicating effects. Single moderate doses of alcohol rarely provide cause for concern in isolation. Consumption of a significant amount of alcohol over a short period however can result in alcohol poisoning that results in death. Additionally, any amount of alcohol is able to significantly impair an individual's ability to perform crucial tasks, in the case of driving this often has serious and fatal consequences. Alcohol not only affects psychomotor skills such as coordination and reaction times, but also impairs cognitive decision-making skills that increase the likelihood of an inebriated individual undertaking risky actions (Cherpitel, 2013).

There are also significant issues of chronic alcohol consumption and the long-term effect on humans. Chronic alcohol impacts upon (but is not limited to) chronic liver disease, cardiovascular disease and diabetes (Shield et al., 2013). These represent significant burdens on Western populations in particular but are also increasingly significant in the developing world.

The impact of ethanol consumption on human health has seen it noted as a priority area of health improvement for the World Health Organisation (WHO). The WHO has estimated that in 2004 3.5% of all deaths caused by chronic diseases were attributable to alcohol. Additionally, best estimates suggest that in 2004 14.5 million disability-adjusted-life-years lost were attributable to alcohol (Shield et al., 2013). Ethanol presents a crucial area of interest in the research areas of physiology, pharmacology and sociology. Gains in the understanding of how ethanol works in each of these spheres has the potential to drastically improve the impact ethanol has on lives world over.

1.12 Ethanol Pharmacology

Ethanol has considerably wide-ranging effects on the physiology of all animals. Animal models, using primarily rodent test species, have allowed the quantification of multiple in vivo ethanol effects. This includes decreased mobility and righting reflexes of rodents in the rota-rod test (Soares et al., 2009), ethanol induced enhanced locomotor activity in rodents (Lessov and Phillips, 2003, Valjent et al., 2010), and the anxiolytic action of ethanol that can also perform as a motivational stimulus when studying its effects in rodents. Most notably studied using elevated plus maze and condition place preference paradigms respectively (Boyce-Rustay and Holmes, 2006).

γ-Aminobutryic acid (GABA) receptors are known to be a key receptor that ethanol has a profound effect upon. Primarily ethanol acts at the ionotropic $GABA_A$ receptor. $GABA_A$ receptors are heteromeric and are composed of several protein subunits which are primarily α(1-6), β(1-3), γ(1-3) and δ subunits, though other subunits types do exist (Kumar et al., 2009). The most common synaptic $GABA_A$ subunit composition consists of two α, two β and one γ unit with the γ subunit position between an α and a β subunit though this receptor subunit combination can also be expressed extrasynaptically (Kumar et al., 2009). On the other hand receptors that contain the δ subunit appear to be expressed exclusively extrasynaptically (Lobo and Harris, 2008).

Ethanol enhances GABAergic transmission in low to moderate doses (3-30 mM) (Lobo and Harris, 2008). Two amino acid mutations in $GABA_A$ receptor subunits results in a loss of ethanol potentiation suggesting that direct binding of ethanol to the $GABA_A$ receptor (Mihic et al., 1997). Further studies have deduced that a binding site between $GABA_A$ α4/6+β3 subunits appears to selectively bind ethanol and mediate its GABA enhancing actions at the receptor (Wallner et al., 2014, Olsen et al., 2014).Ethanol interaction with $GABA_A$ receptors may have some important functional significance regarding the interaction of ethanol and opioidergic systems.

Ethanol also has pronounced effects on voltage-gated calcium channels, in particular L-Type calcium channels (LTC). Ethanol has been shown to both decrease the probability of LTC opening as well as shortening the duration of opening, effects that can result in decreased neurotransmitter release and neurotransmission (Mah et al., 2011, Pietrzykowski et al., 2013).Ethanol has also been shown to chronically up regulate LTC's which may play a role in withdrawal symptoms and alcohol craving leading to relapse (Walter and Messing, 1999).

Additionally, ethanol is also known to bind to G-protein activated potassium channels (GIRKs). GIRKs are known to have a binding site for ethanol in close proximity to the G-protein binding site (Bodhinathan and Slesinger, 2014) that facilitates the open state of the GIRK channel resulting in

potassium efflux from the neuron. Alteration in GIRK channel conductance has been associated with several conditions including, Down's syndrome, Parkinson's ataxia and epilepsy. Ethanol modulation of opioidergic systems may be mediated by ethanol alteration of GIRK transmission and so should not be discounted as a possible pathway or mechanism.

Beyond the above, ethanol has also been shown to bind to N-methyl-d-aspartate receptors antagonistically, as well as facilitate signalling through acetylcholine receptors, though the latter is thought to occur at unusually high ethanol doses (Deitrich et al., 1989). Ethanol has been discussed as interacting with glycine receptors, with α1 glycine subunit knock out mouse exhibiting a decreased sensitivity to ethanol mediated sedation (Aguayo et al., 2014).

Ethanol is not considered a selective drug. However, the major dose of ethanol used in this study (0.3 g/kg) is thought to produce an ethanol concentration of no more than 20 mM (Matson et al., 2013) following administration in mice. This concentration as previously stated is considered by some to remain relatively selective for $GABA_A$ receptors (Lobo and Harris, 2008). Despite this evidence it is necessary to consider that ethanol has many potential methods of action and it would be unwise to categorically rule any out at this time.

There is a large body of evidence that links the endogenous opioid system in the psychopharmacological effects mediated by ethanol (Font et al., 2013) with experimental investigations showing an attenuation or blockade of ethanol and its effects using either opioid antagonists (Oswald and Wand, 2004, Camarini et al., 2000) or MOPr knockout mice (Roberts et al., 2000). With the interaction of ethanol and opioids being sensitive to even the endogenous opioid system, the exogenous application of opioids and alcohols in combination has allowed robust studies not only of interaction but also of mechanistic action.

1.12.1 Ethanol Metabolites

A key area of interest when investigating any effect of ethanol is acetaldehyde. Acetaldehyde is the primary metabolite of ethanol and has been noted as an aversive metabolite. The aversive effects of acetaldehyde lead to the prescription of disulfiram (an inhibitor of acetaldehyde dehydrogenase) as a treatment for alcoholics. Disulfiram inhibits the breakdown of acetaldehyde thus leading to its accumulation and an aversive feeling of nausea and sickness. The aversive effect was intended to act as a negative reinforcement that helped prevent relapse (Peana and Acquas, 2013). Acetaldehyde was not originally thought to be important in the development of reward or addiction to ethanol both for this reason and the notion that the main metabolic enzyme of ethanol, alcohol dehydrogenase, was confined to the liver (Quertemont et al., 2004).

However it has since been shown that alcohol dehydrogenase is present within the brain and that a secondary enzyme brain catalase (a common enzyme catalysing the conversion of hydrogen peroxide to water) is also widely available within the brain and breaks down ethanol (Correa et al., 2008). These findings show acetaldehyde can be present within the CNS following consumption or administration of ethanol (Font et al., 2006, Correa et al., 2008).

While the effect of ethanol on opioid tolerance has been demonstrated, there has been little regard for the potential importance of its metabolites. Acetaldehyde in particular could in fact play an important role in the reinforcing properties of ethanol in the CNS. The aversive potential of acetaldehyde accumulation is potentially an effect that is specific to the periphery and is not, in fact, reflective of the pharmacological contribution of acetaldehyde centrally.

1.13 The Emerging Problem of Gabapentoids and Opioids

Gabapentin and pregabalin are gabapentoids that have served as front-line treatments for neuropathic pain and epilepsy; however, they have been increasingly prescribed for a multitude of other conditions, in particular for anxiety, but also insomnia, migraine, bi-polar and alcohol withdrawal (Gomes et al., 2017, Spence, 2013).

Initial data strongly suggested that both gabapentoids possessed low abuse liability and interaction with other prescription medication, which goes a long way to explain the relative freedom with which gabapentoids have been repurposed and prescribed beyond their original remit (Gomes et al., 2017). However, recent reports indicate that pregabalin and gabapentin are increasingly being abused individually with death occurring in an increasing number of cases (Mersfelder and Nichols, 2016, Schjerning et al., 2016).

Additionally, the concomitant abuse of pregabalin and gabapentin with opioids has been reported in recent years, again with a trend of increasing overdose deaths involving gabapentoids and opioids (Lyndon et al., 2017). This has led to serious concerns, particularly in the UK about gabapentoids and there availability to the general public (Spence, 2013). In fact, gabapentoids have subsequently become scheduled substance within the UK, restricting and controlling the practise of prescribing gabapentoids therapeutically.

1.13.1 Gabapentoid Pharmacology

Pregabalin and gabapentin are analogues of GABA. Despite their analogous structures pregabalin and gabapentin do not bind to either the $GABA_A$ or $GABA_B$ receptors, either orthosterically or allosterically (Calandre et al., 2016, Sills, 2006, Uchitel et al., 2010). Pregabalin and gabapentin have been shown to bind to voltage sensitive calcium channels (VSCCs), specifically binding only to VSCCS that contain the $\alpha_2\delta$ sub-unit (Calandre et al., 2016, Sills, 2006, Uchitel et al., 2010). Pregabalin inhibition of $\alpha_2\delta$ sub-unit containing VSCC is thought to be their primary mechanism to mediate the anti-convulsant, anxiolytic and analgesic effects of pregabalin and gabapentin (Calandre et al., 2016, Sills, 2006, Uchitel et al., 2010).

Gabapentoids may, however, have additional pharmacological properties that are responsible for potentially dangerous synergism with opioid use. Pregabalin and gabapentin use may enhance the effect of acute opioid induced respiratory depression (as well as euphoria), simply act as a CNS depressant in summation with opioids, or like ethanol, pregabalin may interact with opioid tolerance to enhance the dangers of opioid abuse and increase the likelihood of an opioid overdose (Spence, 2013, Lyndon et al., 2017).

1.14 Protein Kinase C and Morphine

1.14.1 Electrophysiological Evidence

Morphine is the prototypical opioid of choice for pain management and the primary metabolite of heroin. This duality has made morphine one of the most scientifically investigated opioids. A large number of these investigations have centred around the down-stream kinases that are recruited following morphine agonism at the MOPr, and how this relates to both morphine induced MOPr desensitization and morphine tolerance (Alvarez et al., 2002, Bailey et al., 2009b, Hull et al., 2010, Johnson et al., 2006, Lowe and Bailey, 2015, Melief et al., 2010). To date, there is substantial evidence that implicates protein kinase C (PKC), and it multiple isoforms, as being the primary kinase responsible for morphine induced desensitization and tolerance (Bailey et al., 2009b, Hull et al., 2010, Johnson et al., 2006, Chen et al., 2013).

Electrophysiological investigation of MOPr desensitization are often conducted in the LC, due the absence of delta and kappa opioid receptors. Prior investigation has shown that incubation of rat brain LC slices in 3μM morphine for 6-9 hours, significantly reduces GIRK channel conductance following the application of morphine (30μM) (Bailey et al., 2009a). As with previously noted investigations (Llorente et al., 2013), GIRK channel conductance following application of NA was unchanged in morphine incubated slices, indicating the reduction of GIRK conductance seen following morphine (30μM) application, was MOPr homologous desensitization.

However, addition of Gö6976, a potent, broad spectrum PKC inhibitor, 15-30 minutes prior to morphine (30μM) in tolerant LC slices was able to significantly reverse MOPr desensitization induced by morphine incubation (Bailey et al., 2009a). In these experiments Gö6976 did not alter morphine GIRK currents in control slices. This finding was replicated in LC slices taken from rats that had received a 3-day treatment of slow release formula morphine (200 mg/kg). In these experiments, desensitization was homologous to the MOPr when compared to NA responses, and MOPr desensitization this was significantly reversed by the application of the PKC inhibitor Gö6976.

However, when a concentration of DAMGO (30nM) that was equipotent with morphine (30μM), or a receptor saturating concentration of DAMGO (10 μM), was applied to LC slices for 10 minutes, the following addition of 30μM morphine produced profound MOPr desensitization. PKC inhibition by Gö6976 was not able to reverse the desensitization to morphine back to control levels in these experiments.

As well as electrophysiological evidence of morphine dependent MOPr desensitization being reversed by PKC inhibition (Bailey et al., 2009a), there is also evidence of the converse. That is to say that PKC activation can enhance MOPr desensitization to morphine. A 7 minute application of morphine (30 $\mu mol.L^{-1}$) has been shown insufficient to produce desensitization at the MOPr in LC brain slices from mature rats (Bailey et al., 2009b). However concomitant activation of M_3 muscarinic receptors in LC slices by the addition of oxotremorine-M (oxo-M) is known to enhance PKC activity (Bailey et al., 2004).

The addition of oxo-M (10 $\mu mol.L^{-1}$) before the 7 minute application of morphine was found to induce significantly greater MOPr desensitization. This has also been shown previously using the Phorbol ester Phorbol 12-myristate 13-acetate (PMA) to activate PKC (Bailey et al., 2004). This oxo-M mediated increase in morphine desensitization of the MOPr was completely inhibited by the addition of the PKC inhibitor Gö6976. In contrast, neither PKC activation nor PKC inhibition had any effect on MOPr desensitization induced by a receptor saturating concentration of DAMGO (10 $\mu mol.L^{-1}$) (Bailey et al., 2009b).

Bailey et al., (2009b) also utilised receptor for activated C-kinases (RACK) inhibitors. RACK inhibitors can specifically inhibit single PKC isoforms, compared to the broad-spectrum inhibition of PKC by Gö6976. Utilising RACK inhibitor specificity, PKCα was found to be the dominant PKC isoform able to inhibit oxo-M enhancement of morphine desensitization. This was then replicated in LC slices from PKCα knockout and wild-type matched mice. In PKCα knockout slices, application of oxo-M was unable to enhance the desensitization of morphine at the MOPr.

This collation of electrophysiological data show that not only is PKC activation able to enhance morphine desensitization at the MOPr, but PKC inhibition is also able to reverse acute morphine desensitization at the MOPr as well as reverse established morphine tolerance at the MOPr. These effects appear to be mediated by the specific PKC isoform PKCα. PKC also appears to only be a crucial kinase in the development of desensitization and tolerance to morphine, as both the acute effects of DAMGO and tolerance induced by DAMGO, remained unaffected by PKC activation or inhibition.

1.14.2 *In vivo* Evidence

Antinociceptive tolerance to opioids, following repeated or prolonged administration of an opioid, is well established in rodents. Use of the warm water tail flick technique is a very common method of measuring the nociceptive spinal reflex in mice, and subsequently the antinociceptive properties of opioids. Previously reported work (Hull et al., 2009), demonstrated that tolerance to the opioid agonists, meperidine, morphine, fentanyl and DAMGO can be rapidly induced.

This was achieved through an injection of each opioid every 2 hours (sc) for 6 hours (a total of 4 injections) with the agonists meperidine (40 mg/kg), morphine (10 mg/kg) and fentanyl (0.2 mg/kg). For DAMGO, a single injection (25.7 ng/kg icv) once per hour for 8 hours (a total of 8 injections) was used. These tolerance induction protocol resulted in a rightward shift of the dose response curve for each agonist, ranging from a 2.4 to a 4.6-fold rightward shift. Injection of the PKC inhibitor Gö6976 (4 nmol/mouse icv) immediately before opioid agonist injection was able to fully reverse tolerance to the relatively low efficacy agonists meperidine, morphine, and fentanyl. However, PKC inhibition had no effect on DAMGO tolerance

Conversely the injection of β-adrenergic receptor kinase 1 inhibitor (β-ARK 1) (20 nmol/mouse icv), a GRK inhibitor, was able to fully reverse DAMGO tolerance, but had no significant effect on tolerance to meperidine, morphine or fentanyl. Whole-cell voltage clamp experiments in mouse LC slices showed that application of DAMGO (1-10μM) produced significant desensitization over 15 minutes. This was reversible through application of the GRK inhibitor β-ARK 1 but not by the PKC inhibitor Gö6976.

In agreement with the electrophysiological data described previously, Hull et al demonstrated that DAMGO induced desensitization and tolerance is distinct from that of morphine desensitization and tolerance at the MOPr with the former operating through a PKC insensitive mechanism. However, Hull et al (2013), advance this one step further, by suggesting that the ability of PKC inhibition to decrease physiological tolerance to both meperidine and fentanyl as well as morphine, is a factor of their efficacy at the MOPr. This would suggest that opioid agonist efficacy is one of the determining factors for selective kinase activity following agonist activation of the MOPr.

Further work has demonstrated that not only can PKC inhibition, diminish or entirely reverse opioid tolerance, but that it can also enhance the basal antinociceptive properties of acute morphine in naïve animals (Galeotti et al., 2014). St. John's Wort has been reported to inhibit PKC activity through its main active component, hypericin (Takahashi et al., 1989, Kocanova et al., 2006).

Galeotti et al, first demonstrated that hyepricin (0.15µg/mouse icv) was able to potentiate the antinociceptive effect of morphine in mice using the hot plate test. The hotplate involves placing the mouse upon a temperature regulated plate that noxiously heats the paws of the animal. The time taken to lick the paws, in order to cool them, is taken as a measure of nociception. An analgesic will increase the latency to paw lick. The hypericin induced enhancement of acute morphine antinociception was replicated following administration of calphostin C, a none isoform-specific PKC inhibitor. This suggests a basal level of PKC activity which presents an active block to morphine antinociception even in opioid naïve mice.

Following this, mice were rendered tolerant to morphine through a 4-day escalating dose regime (10 mg/kg – 15 mg/kg – 20 mg/kg – 30 mg/kg twice daily sc). The hotplate test was used to assess tolerance. In tolerant mice, morphine (5 mg/kg) did not produce a significant antinociceptive response compared to control. The antinociceptive response to morphine was significantly enhanced in tolerant mice that were also administered hypericin (0.15µg/mouse icv). Hypericin (ICV) also enhanced basal morphine antinociception in the hot plate test.

Hypericin was also shown to enhance the analgesic effect of morphine in healthy volunteers co-administered hypericin and morphine. Nociception was measured by immersion of the dominant arm in noxiously warm (46 degrees Celsius) water up to and including the elbow. Subjective pain measurements were taken every 30 seconds for 2 minutes. Volunteers administered both oral morphine (10 mg/5ml) and hypericin (9mg) showed significantly decreased pain scores compared to morphine alone.

1.15 The Role of PKC and Ethanol in Opioid Tolerance

Both in vitro and in vivo data present strong evidence for the role of PKC in opioid tolerance being a significant one. However, PKC appears to be recruited in a ligand selective manner applied (Bailey et al., 2009b, Johnson et al., 2006). The intrinsic efficacy of the opioid ligand has been suggested as the defining factor for PKC activation following agonist activation of the MOPr (McPherson et al., 2010). Tolerance and desensitization induced by lower efficacy agonists such as morphine, appear to be far more susceptible to manipulation by PKC activation or inhibition. This is in stark contrast to the high efficacy agonist DAMGO. DAMGO induced desensitization has been shown to be insensitive to inhibition or potentiation of PKC activity (Bailey et al., 2009b, Johnson et al., 2006)..

The correlation of PKC inhibition and ethanol application on reversing morphine induced desensitization and tolerance, but not on DAMGO induced tolerance, suggests that these phenomena may work through the same molecular pathway, or at least interconnecting molecular pathways. Ethanol may affect reversal of morphine tolerance through the inhibition of PKC. This may be particularly observable in tolerant animals due to an upregulation of PKC activity following the development of tolerance to prolonged morphine exposure.

1.16 Intrinsic Agonist Efficacy for G-protein – GRK Signalling: A Predictor of Susceptibility to Reversal by Ethanol

Previous work has demonstrated that MOPr desensitization can be induced through either G-protein or GRK dependent signalling pathways, dependent on the MOPr agonist applied (Bailey et al., 2009b, Johnson et al., 2006). Additionally, previous work has shown that morphine but not methadone induced tolerance *in vivo*, is susceptible to reversal by ethanol (Hill et al., 2016).

The intrinsic efficacy of a range of opioid agonists to signal through G-protein or GRK at the MOPr has previously been investigated (McPherson et al., 2010). McPherson et al (2010) demonstrated that morphine has a relatively low intrinsic efficacy to signal through GRK compared to a high intrinsic efficacy of methadone to signal through GRK. Methadone has a similar intrinsic efficacy for GRK as DAMGO, which has been previously shown to induce MOPr desensitization through a GRK dependent mechanism.

Given that both methadone tolerance in vivo and DAMGO desensitization of the MOPr in vitro are not reversible by ethanol, and both display high intrinsic efficacy for the GRK signalling pathway, one might hypothesise that tolerance induced by opioid agonists that display high efficacy for GRK signalling in general will be less susceptible or not susceptible at all to reversal by ethanol, and conversely tolerance induced by opioid agonists with low intrinsic efficacy for GRK signalling such as morphine and oxycodone will be susceptible to reversal by ethanol.

1.17 Hypotheses

The primary hypotheses in this thesis are:

I. Tolerance to morphine respiratory depression is induced by oxycodone.
II. Pregabalin reduces the level of opioid tolerance, dependent on the agonist used to induce tolerance.
III. PKC inhibition reduces the level of opioid tolerance, dependent on the agonist used to induce tolerance.
IV. The intrinsic efficacy of an opioid agonist for G-protein vs GRK predicts whether tolerance induced by an opioid agonist can be reversed by ethanol, pregabalin or PKC inhibition.
V. Fentanyl induced respiratory depression is resistant to naloxone reversal.

2.0 Materials and Methods

2.1 Ethical Considerations

Due to the use of animals in scientific experimentation, all due ethical consideration must be given to the well-being and welfare of all animals involved. All procedures and experimentations in this thesis were performed in accordance with the UK Animals (Scientific Procedures) Act 1986, the European Communities Council Directive (2010/63/EU) and the University of Bristol ethical review document.

2.2 Animals

Adult male CD-1 mice (Harlan UK) aged 4 weeks and weighing 21-25g upon arrival were used in all *in vivo* experiments. They were housed for at least 5 days in groups of 4-8 before use. Each cage was provided with sawdust, bedding and a single enrichment item provided in the form of a cardboard tube. Cages were maintained in a 21-24^{o}C and 40-60% humidity environment, on a reversed light cycle, with the dark cycle running 8:00am to 8:00pm. All experiments were conducted in the dark cycle (active phase) under red light conditions. Mice were transported in cages in an opaque bag through any lit corridors and rooms. The average experimental group weight was approximately 30g at the beginning of each experiment.

2.3 Drugs

Morphine hydrochloride (Macfarlan Smith), oxycodone hydrochloride (Sigma Aldrich UK), methadone hydrochloride (Sigma Aldrich UK), fentanyl citrate (Sigma Aldrich UK), and ethanol (Sigma Aldrich UK - 100%) were all made up in solution to an appropriate dose using sterile saline (0.9% NaCl). Calphostin C (Sigma Aldrich UK) SP600125 (Tocris Bioscience UK), and Compound 101 (University of Bath UK) were dissolved in 100% dimethyl sulfoxide (DMSO) before being diluted with sterile saline, such that the final concentration of DMSO was 1%. All injections were given intraperitoneally (i.p.) in a volume of 0.1 ml. 75mg Morphine pellets were kindly provided by the National Institute of Health (USA).

2.4 Induction of Opioid Tolerance

2.4.1 Implantation of Prolonged Opioid Delivery Systems

In order to achieve a relatively prolonged delivery of opioids to mice over a period of up to 6 days, surgical implantation of either an osmotic mini-pump with an opioid solution filled reservoir, or a 75mg morphine pellet was performed. Surgical implantation of pumps or pellets were performed under anaesthesia as described in Materials and Methods section 2.5.

2.4.1.1 Implantation of Morphine Pellet

Morphine free base pellets weighing 75mg were kindly provided by the National Institute of Health, National Institute of Drug Abuse (Bethesda, MD, USA). Morphine pellets were surgically implanted subcutaneously on the dorsal flank of mice for a period of no more than 6 days in order to produce tolerance to opioid induced antinociception and opioid induced respiratory depression.

2.4.1.2 Implantation of Osmotic Mini-pumps for Prolonged Opioid Administration

Osmotic mini-pumps were procured from Alzet (supplied by Charles River, UK). The size of pump used was 2001D which is designed for use in mice with a minimum body weight of 20g required for implantation. These pumps had a flow rate of 1µl/hour from a 200µl reservoir and provided enough opioid solution for a 7 d administration. Osmotic mini-pumps were surgically implanted subcutaneously on the dorsal flank of mice for a period of no longer than 6 days. All pumps were filled under sterile conditions according to the manufacturer's guidelines.

Previous research using Alzet osmotic mini-pumps to administer methadone described the use of pre-injections prior to pump implantation to enhance the development of tolerance (Quillinan et al., 2011). The pre-injections described consisted of three separate injections given 12 hours apart with the last injection occurring 12 hours before surgical implantation of the osmotic mini-pumps. In this thesis, the pre-injection protocol was adapted for all opioids. Pre-injections were given on alternating sides of the peritoneal cavity to minimise bruising and suffering of the mice.

2.4.1.3 Pump Preparation

For all pump implantations, pre-injections were given 12 hours apart in 0.1 ml. 12 Hours after the final injection surgical implantation of osmotic mini pumps was performed as described in Materials and Methods section 2.5.

2.4.1.4 Morphine Pump Protocol

Three x 100 mg/kg morphine injections followed by implantation of an osmotic mini-pump containing 200µl of 56.25 mg/ml morphine. This provided 45 mg/kg/day morphine.

2.4.1.5 Oxycodone Pump Protocols

Three schedules of oxycodone treatment were used:

(i) Low dose treatment – 3 x 30 mg/kg i.p injections followed by implantation of an osmotic mini-pump containing 200µl of 25 mg/ml oxycodone. This provided 20 mg/kg/day oxycodone.

(ii) Moderate dose treatment – 3 x 100 mg/kg i.p injections followed by implantation of an osmotic mini-pump containing 200µl of 56.25 mg/ml oxycodone. This provided 45 mg/kg/day oxycodone.

(iii) High dose treatment – 3 x 100 mg/kg i.p injections followed by implantation of an osmotic mini-pump containing 200µl of 150 mg/ml oxycodone. This provided 120 mg/kg/day oxycodone.

2.4.1.6 Methadone Pump Protocol

One x 5 mg/kg methadone hydrochloride injection was given to animals followed by two x 7.5 mg/kg methadone injections, followed by implantation of an osmotic mini-pump containing 200µl of 75 mg/ml methadone. This provided 60 mg/kg/day methadone.

2.4.2 Induction by Multiple Injections Protocol

Multiple injections protocols to induce tolerance were used in lieu of osmotic pump or morphine pellet implantation in several experiments.

2.4.2.1 Multiple Morphine Injections

Multiple morphine injections were administered as twice daily doses of morphine 10 mg/kg, with each dose given 12 h apart by i.p. injection. This was conducted for a total of 5 days.

2.4.2.2 Multiple Fentanyl Injections

Two doses of fentanyl (0.15 mg/kg) were administered i.p. 2 h apart. This protocol was adapted from previously published that the acute administration of two doses of fentanyl (0.3 mg/kg) given 3 hours apart can produce a significant degree of tolerance to the antinociceptive effect of the second dose (Melief et al., 2010).

2.5 Surgical procedure

Animals were anaesthetised using 2.5-3% isoflurane in oxygen in a mouse anaesthetic box. No pre-operative or post-operative analgesics were administered. Following loss of consciousness, the head of the mouse was placed carefully in a nose cone unit providing anaesthetic with an appropriate scavenging unit collecting excess anaesthetic gas. Cessation of nociceptive reflexes was determined by both a tail pinch and a foot pinch before conducting any further part of the procedure. The hair around the base of the neck was shaved to provide an area of clear skin. A 1-1.5 cm lateral incision was made in the skin at the base of neck and using a pair of straight forceps a subcutaneous pocket was formed on the dorsal flank of the mouse.

When a 75 mg morphine pellet was implanted, it was inserted in the pocket so that it sat above the base of the tail.

For Alzet osmotic mini pumps filled with 200µL of either morphine, oxycodone or methadone solution, the pumps were inserted subcutaneously to sit above the spine of the mice with the release end of the pump inserted first pointing towards the tail. The same method of incision as for implantation of pellets was used for implanting the pumps.

All incisions were closed using Clay Adams Brand, MikRon Autoclip 9-mm Wound Clips (Harvard Instruments) and the wound treated with wound powder (Hayward and Bower). The entire surgery took approximately 5 min. After cessation of anaesthetic administration mice were monitored in a recovery cage until they demonstrated normal exploratory behaviour and righting reflex when the cage was gently tipped. Following full recovery mice were placed back into their own home cage. Mice were checked 8-12 h after surgery with additional wound powder applied to the site of incision to ensure no infections occurred.

Mice recovering from surgery were never placed in a cage with mice that had not undergone surgery. Nor did mice of one cage ever receive mixed opioid treatments.

2.6 Monitoring respiration in freely moving mice

Plethysmograph chambers were purchased from EMKA Technologie (France). Four chambers were set up with differential pressure transducers and temperature/humidity monitor. These were connected to either a four-way air pump circulating room air through the chambers or to a mass flow controller enabling the delivery of a 95% air/5% CO_2 (non-humidified) gas mixture. Both air and gas mixtures were delivered at 0.5 L/min. Chambers were calibrated at this flow rate using a 1mL injection of air via a syringe to provide an adequate volume range in order to detect mouse inspiration and expiration. Calibration was performed no less than once every 2 months to ensure consistency of data. Whilst in chambers mice were restricted from eating but drinking water was freely available. Chambers were thoroughly cleaned and dried between each animal.

Each plethysmograph chamber is fitted with a differential pressure transducer (DPT). A DPT allows a comparison of the atmospheric pressure within the experimental room and the intra-plethysmograph chamber pressure. When a mouse was placed within the chamber the DPT is able to take the analogue pressure changes which occur as a result of mouse respiration. As a mouse inspires this produces a relative decrease in pressure and as such this is recorded by the DPT as a negative change in chamber pressure and a downward deflection of the respiratory trace.

Conversely the exhalation of a mouse produces a relative increase in chamber pressure and a positive pressure change which is illustrated as an upward deflection on the respiratory trace. The point of inflection on the respiratory trace represents the point within a respiratory cycle where the atmospheric and chamber pressure are equal.

Figure 2.1 illustrates a typical respiratory trace in air with inspiratory volume indicated by **A** and expiratory volume indicated by **B**. The combination of these two areas provides a full respiratory cycle, with respiratory rate determined by the number of full cycles (indicated by **C**) in a minute. Tidal volume is the volume of air inspired in a single cycle i.e. **A** + **B**. The central horizontal line indicates 0 volts where inspiratory and expiratory pressures are equal. Minute volume (MV) was calculated using tidal volume X number of breaths.

As chambers were a confined space with an approximate volume of 800cm^3 mice were habituated to the chamber the day prior to the experiment. Mice were habituated for 30 min no more than 18 hours prior to experimentation.

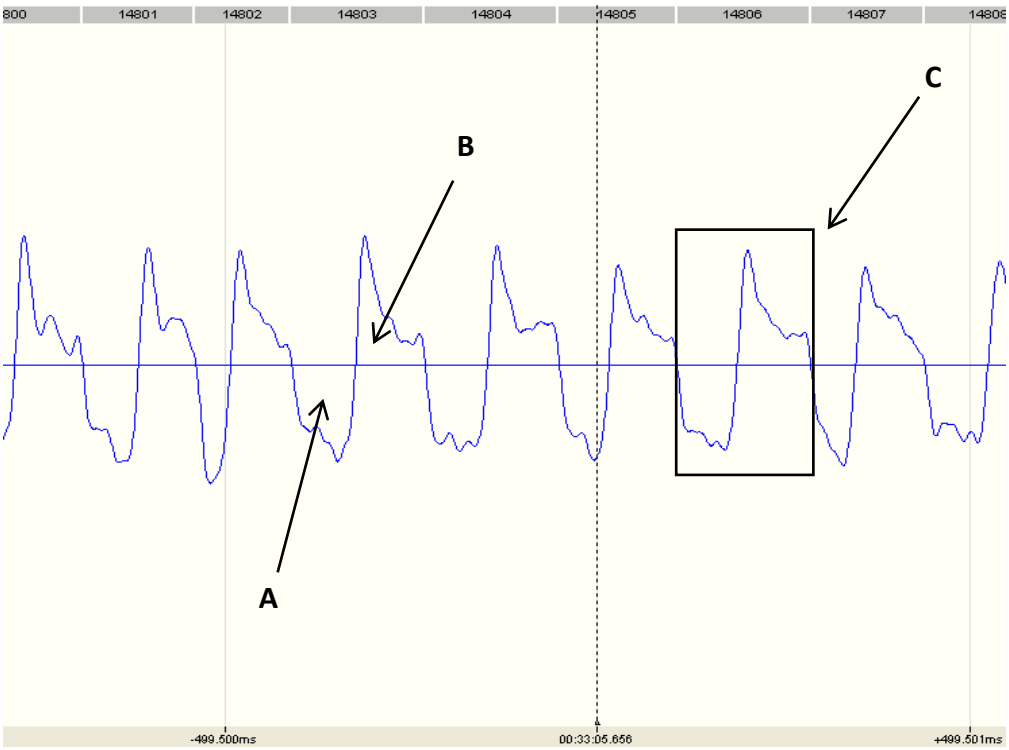


Figure 2.1. Typical mouse respiratory trace in air. A indicates i*nspiratory volume which is represented by a downward deflection in the trace. **B** indicates expiratory volume which is represented by an upward deflection in the trace. **C** is a single respiratory cycle, the number of these in a minute indicates number of breaths per minute (BPM). **A**+**B** = Tidal volume (TV). **TV X BPM** = Minute Volume.*

2.7 Analysis of Respiratory Waveforms

A breath is detected when consecutive minimum (Peak inspiratory flow PIF) and maximum (Peak expiratory flow PEF) values exceed a pre-set defined Flow Threshold (3ml/s). Recommended Flow Threshold is 6ml/s, however a lower flow threshold has been used due to the nature of investigating decreased respiratory parameters induced by opioids.

The beginning of the breath is determined by inspiration interpolation. This is defined by 2 points on the curve that precedes PIF. These points are defined as 20% and 40% PIF. These points then define a line along the inspiration curve that crosses 0 flow. The point of 0 flow is defined as the start of the breath. The end of inspiration (or the start of expiration) is then defined as when the waveform crosses 0 flow again.

The end of expiration is then defined as the point at which the waveform crosses the 0 flow point sequentially after PEF. Tidal volume is calculated by integration of the flow from the start of inspiration to the end of inspiration (inspiratory volume IV) and the start of expiration to the end of expiration (expiratory volume EV). These EV and IV values are compared to the pre-set allowance for deviation between these integrated areas (30%). If the IV and EV values deviate less than the pre-set, then the breath is accepted as a valid respiratory event. A degree of deviation is required, due to the humidification of inspired air within the lung, which alters the pressure released during expiration.

Breathing rate is defined as the number of PEF to PEF points that have been accepted as valid respiratory events.

2.8 Respiration Experiments

2.8.1 Measurement of Acute Opioid Respiratory Depression

The day prior to experimentation mice were habituated to plethysmograph chambers for 30 min whilst breathing room air supplied by a pump at 0.5L/min. On the day of experimentation, the plethysmograph chambers were supplied with a dry gas canister mix of air + 5% CO_2 supplied at 0.5L/min. Baseline respiration, taken as the mouse's minute volume, was measured for 20 min in 5-min bins of data. Following the measurement of baseline respiration, mice were removed from the plethysmograph chambers one at a time and scruffed. Whilst scruffed mice received an i.p. injection of drug or vehicle in 0.1ml volume.

Immediately following injection mice were placed back in the plethysmograph chambers. All mice were injected within a 5-min period i.e. the 20-25 min period of the experiment. After the 5-min injection window, respiration was monitored for a further 30 min, also in 5-min average bins of data. Following the end of the experiment, mice were removed from the plethysmograph chambers and culled by destruction of the brain followed by cervical dislocation.

2.8.2 Measuring the Onset of Tolerance to Opioid Respiratory Depression

In order to measure the development of tolerance to opioid-induced respiratory depression, where the opioid is provided solely by a subcutaneously implanted osmotic mini-pump or 75mg morphine pellet, a baseline measure of mouse MV was required. Mice were habituated to a plethysmograph chamber for 30 min whilst breathing room air supplied by a pump at 0.5L/min. On the following day mice were placed in the plethysmograph chambers for 20 min. During this 20 min the chambers were supplied with a dry gas canister mix of air + 5% CO_2 supplied at 0.5L/min.

This baseline measure of minute volume was taken on the day immediately prior to the animal's first exposure to any opioid. In the case of an osmotic mini-pump delivering buprenorphine or a 75mg morphine pellet this occurred on the day immediately prior to surgical implantation.

In the case of osmotic mini-pumps delivering either methadone or morphine, the baseline MV measurement was made the day immediately prior to the first priming injections of opioids that preceded pump implantation.

Mice had their tails marked with permanent marker with ascending numbers of lines perpendicular to the tail. This ensured that each mouse could be placed in a different chamber on different days during the prolonged treatment and still have each measurement of respiration tracked individually rather than as a group average. Tail markings were re-applied as necessary when fading occurred. Measurement of baseline respiration for 20-min repeated daily for 6 d.

2.8.3 Measuring Tolerance to Acute Opioid Respiratory Depression

On day 6 of opioid treatment mice had their 20-min baseline respiration measured. This was followed by an acute injection of a challenge opioid (typically morphine or other opioid drugs), with each mouse removed from the plethysmograph chambers in turn and injected intraperitoneally within a 5-min window. Respiration was monitored for a further 30 minutes following injection. Following the end of the experiment, mice were removed from the plethysmograph chambers and culled. If a mouse had received a 75mg morphine pellet as a prolonged form of opioid administration, this was removed from the cadaver. This allowed the remaining morphine within the pellet to be disposed of in line with the University of Bristol and Home Office guidelines regarding the use and disposal of controlled, scheduled substances.

2.8.4 Measurement of Reversal of Tolerance to Opioid Respiratory Depression

In order to examine the ability of additional drugs of abuse or kinase inhibitors to reverse opioid tolerance, minor changes were made to the protocol previously described. When examining the ability of a given drug to reverse tolerance to opioid induced respiratory depression, these drugs were injected either concomitantly with the injection of an opioid challenge or given 30 min prior to the opioid challenge. Control groups received saline injections at the same time as experimental drugs were administered. Experimental drugs or saline injections were injected into the opposite side of the peritoneal cavity to the administered opioid to minimise the amount of pain suffered by the mouse. Experimental drugs were also administered alone to naïve mice, to ensure they did not themselves alter respiration.

2.9 Analysis of Respiratory Data

Mouse respiration was measured as minute volume. Due to variability between the baseline minute volume of different experimental groups, the effect of a given drug, or drug combination, may be affected if only the change in minute volume were analysed. Additionally, analysing only group data may hide the responsiveness of mice that started at relatively higher or lower respiratory baselines.

In order to analyse fully the degree of respiratory depression seen, each mouse's baseline minute volume respiration acted as its own control value and the minute volume recorded following drug administration was then expressed as a percentage of the baseline value. Thus, each mouse had their respiratory response to any given drug normalised to their own individual pre-drug baseline. Final X/Y plots of respiratory data are shown as "Percentage of baseline minute volume (%) over time post drug injection".

2.9.1 Example of Percent of Baseline Minute Volume Calculation

The following Table 2.1 provides an example using model data to illustrate how the post-drug data for minute volume (MV) will be expressed in the results sections of this thesis:

Time (min)	Model Data (MV)	Calculation	Explanation
5	130	-	First bin not included in baseline as stabilisation of MV can vary
10	160	$\frac{160+140+150}{3} = 150$	The three 5 min bins are averaged as a stable baseline
15	140		
20	150		
25	170	-	The 25 min bin represents the injection period and has the disturbance of chamber opening/closing
30	75	(75/150)*100 = 50%	The MV, post injection, is divided by the baseline and multiplied by 100 to provide a % of baseline

Table 2.1: Example Calculation of Percent of Baseline Minute Volume. *An illustration of how baseline minute volume is calculated and how this acts a reference point for changes in minute volume that occur after drug injection. A full data set would extend till the 55 min point.*

2.9.2 Analysis of Percent of Baseline Minute Volume Data

In order to analyse the overall effect of an opioid or other drug on minute volume as a measure of respiration over the whole 30 min post drug period, area under the curve (AUC) analyses were performed on the "Percentage of baseline minute volume (%) over time post drug injection" data. Performing this analysis allowed smaller but sustained effects of drugs on respiration to be quantified more easily. In order to calculate the AUC for each group, data from each mouse were plotted as a single line in an X/Y plot with the same defined axis as the group plotted data. This allowed the AUC for each individual mouse to be calculated (Fig. 2), allowing the group average AUC to be plotted as a histogram.

To calculate the AUC, a response of 100 was set as the reference point. This represented a mouse respiratory trace that did not change at all from baseline respiratory values; i.e. over the 30 min post drug period this reference mouse stayed at 100% of its baseline respiration. The difference between this reference line and each mouse's individual respiratory response over the 30 min post drug period could be calculated. This is illustrated in Figure 2.2. The AUC values produced from this calculation were therefore:

AUC = Time (measured as minutes post drug) X Percentage Change in Baseline respiration

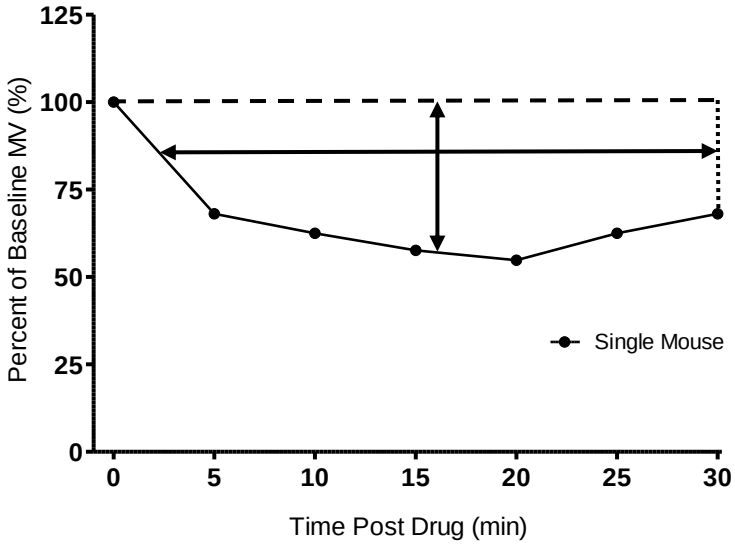


Figure 2.2: Calculation of Area Under the Curve for Percent of Baseline MV*. A single post-drug percent of baseline MV response (closed circles) is illustrated. The dotted line represents the end point of the experiment and the dashed line represents the standardized reference line of 100% i.e. zero change in minute volume from baseline. The area indicated by the double arrows represents the area measured and determined by an area under the curve calculation.*

Each mouse's individual AUC could then be collated back into a group to provide a histogram with error bars that would then be in a format appropriate for statistical analysis. If an AUC calculation had been performed on the group plotted X/Y data, then only a single AUC value would have been presented and the variance within the group lost.

2.10 Measurement of Nociceptive Reflex

A self-regulating water bath maintained at 52^{o}C was used to assess spinal reflexes of mice following thermal nociceptive stimulation of the tail. Mice were scruffed firmly in the right hand and their flaccid tail placed no more than 2 cm under the surface of the water until a tail-flick was elicited. The time from insertion of tail to removal i.e. the tail flick latency was measured. A 20 second (52^{o}C) cut off was used in all experiments in order to prevent thermal damage to the tails of mice. With mice restrained via scruffing to allow dipping of tails, the free hand was used to operate the timer. Measurements were made no more frequently than every 15 min to minimise the chance of permanent thermal damage to tails.

Tail flick data are represented either has latency to tail flick (in seconds) or as a percentage of the maximum possible effect (%MPE). %MPE Is calculated as follows:

%MPE = (Post drug latency – baseline latency) / (Cut-off latency – baseline latency) X 100%

2.11 Nociception Experiments

2.11.1 Acute Antinociception

In order to measure acute antinociception a baseline nociceptive response was required. In order to obtain a stable baseline reading, three separate measures of the tail flick response of mice were made, each 15 min apart. Following the third measurement, an experimental drug was administered by i.p. injection. The tail flick response was then measured at 15 min intervals for 90 min after. This provided a total of 5 post injection measurements of the nociceptive reflex.

2.11.2 Tolerance to Opioid Antinociception

When examining the development of tolerance to opioid antinociception, that arose from the prolonged administration, baseline tail flick latency was measured the day before any opioid administration started, and then every day thereafter. Each day's measurement consisted of measuring three time points each 15 min apart that were then averaged to give the response latency for that day.

2.11.3 Reversal of Tolerance to Acute Antinociception

To investigate the ability of drugs to reverse tolerance to opioid antinociception, the timing of the injection of these drugs, relative to the acute opioid challenge, changed the protocol for the measurement of antinociception. If the experimental drug was to be given concomitantly with acute opioid challenge, then the protocol was not changed. However, certain drugs required a 30 min pre-treatment before the acute opioid challenge. In these cases, additional baseline measurements were made at -60 min, and -45 min, relative to the acute opioid injection (Fig. 3). Following the -30 min measurement of the tail flick response, the experimental drug was injected intraperitoneally for a 30 min pre-treatment period. This allowed measurement of tail flick at -15 and 0 min to determine whether the experimental drug itself had any antinociceptive or pro-nociceptive effect, before the acute opioid dose was administered immediately after the 0 min measurement. This is illustrated in Figure 2.3.

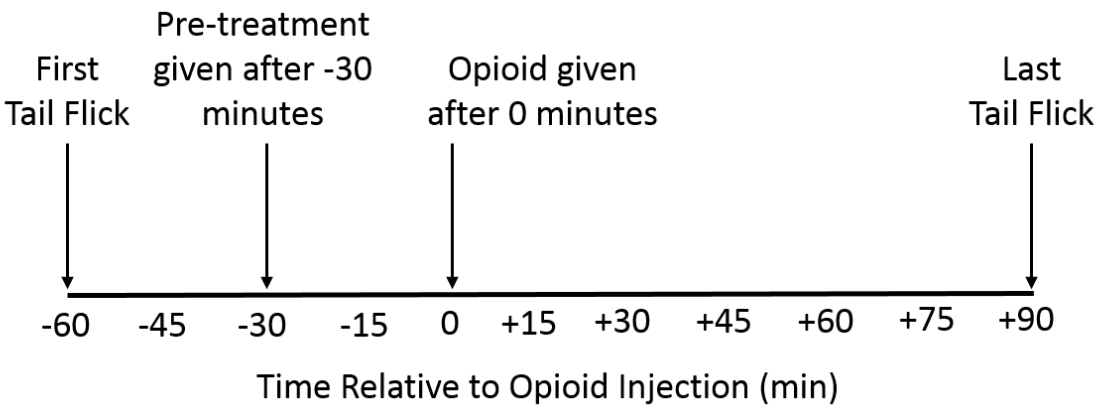


Figure 2.3: Timeline of Tail Flick experiments that require a drug pre-treatment. *Each time point represents a measurement of tail flick latency taken 15 min. Measurements taken following drug pre-treatment allow analysis of changes in tail flick latency due to pre-treatment before injection of an opioid.*

2.11.4 Acute Antinociception Tolerance

Previously it has been reported that two doses of opioid given in close temporal proximity (2-3 h) can produce acute tolerance to opioid antinociception (Melief et al., 2010). Melief et al, demonstrated that fentanyl (0.3 mg/kg i.p.) produced a significant antinociceptive response that returned to baseline after 3 h; following return to baseline a second injection of the same dose of fentanyl produced significantly less antinociception Figure 2.4. This protocol of acute antinociceptive tolerance was replicated in this thesis using acute fentanyl (0.15 mg/kg) or morphine (10 mg/kg). Additionally, different drug pre-treatments were given, in an attempt to prevent or reverse tolerance, these will be described in the individual results sections.

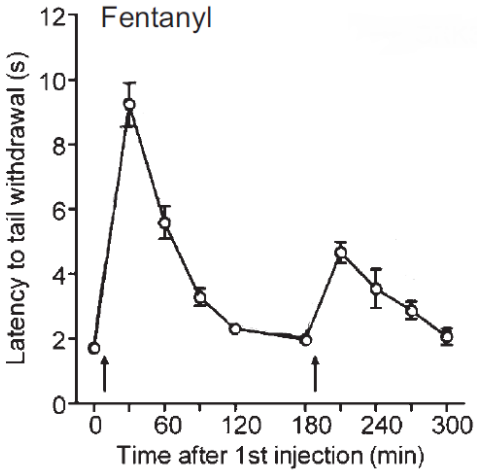


Figure 2.4: Acute repeated doses of fentanyl produce acute antinociceptive tolerance: *Melief et al demonstrated that acute fentanyl (0.3 mg/kg) administered at time points indicated by the upward arrows produces a significant decrease in antinociception seen following the second dose (open circles). Figure adapted from Melief et al., 2010.*

2.12 Measurement of Both Opioid Induced Antinociception and Respiratory Depression

The majority of nociception and respiration experiments were conducted on different batches of mice, however there were some occasions in which it was advantageous to record both of these parameters in the same mice such that a mouse could be both its own control for both opioid antinociception and opioid respiratory depression. In these experiments the tail flick nociceptive baseline was measured prior to the mice being placed in the plethysmograph chamber on the experimental day.

This baseline was made up of three separate measurement separated by 20 min intervals. Mice were habituated to the plethysmograph chamber as described previously. Following the 20 min baseline measurement of respiration mice were administered an opioid in a 5 min injection window before being placed back in the plethysmograph chamber and respiration was monitored for a further 20 min after the 5 min injection window. Following removal from the plethysmograph chambers, the tail flick latency of mice was measured before the mice were returned to the plethysmograph chamber. This was then repeated a further four times over an hour with each tail flick measurement made 15 min apart.

2.13 Prolonged Ethanol Diet Administration

To investigate the effect of prolonged ethanol on the development of opioid tolerance, a liquid diet was used to administer control diet or 5% ethanol diet to 4 groups (two groups of each diet) of mice for 2 weeks. The premise for using both a 2 week administration and a 5% volume of ethanol was based upon (Bertola et al., 2013) who demonstrated the exact amount of dietary components required, based on the Lieber-DeCarli '82 diet, to administer doses of ethanol ranging from 1 – 5% over a period of 2-6 weeks.

A two-week period with a 5% ethanol diet was chosen as it was demonstrated by Bertola et al (2016) that this length of exposure and ethanol concentration produced an appreciable blood ethanol content in mice (approximately 180 mg/dL), yet many of the stereotypical hallmarks of liver and kidney damage such as liver steatosis and elevated levels of serum alanine transaminase and aspartate aminotransferase were very mild or entirely absent. This would allow us to minimise the impact of metabolic dysfunction in the experiment whilst using an established ethanol administration protocol with confidence in producing a significant blood ethanol content compared to control diet fed mice.

The Lieber-DeCarli '82 diet used in this experiment was purchased as control (F1259 SP) and ethanol (F1258 SP) dry mix from Bioserv (USA). Maltose Dextrin was purchased commercially (Sigma Aldrich). Following arrival, all liquid diet components, with the exception of ethanol, were stored at 4-8^{o}C as per supplier datasheet instructions. Liquid diet components were not used past the specified use by dates.

2.13.1 Diet Constitution

The liquid control and ethanol diets were made up fresh for each day of the experiment. The volume made up each day of each diet was 330ml. Each cage was provided with 110 ml. Table 2.2 provides the exact constituent amounts required to make 330ml of each formulation.

Diet	Ethanol (%)	Dry Mix (g)	Maltose Dextrin (g)	Ethanol (ml)
Control	0	76	-	-
Ethanol	5	44.3	6.8	16.5

Table 2.2. Composition of Control and Ethanol Diets. *The ethanol diet required the addition of maltose dextrin to remain calorie consistent with the control diet.*

Approximately 200 ml of water was added to a sealable mixing flask before each dietary component was weighed out and added to the liquid. Following the addition of all dietary components, the mixture was made up to a final volume of 330ml before the flask was sealed. The flask was shaken thoroughly for as long as required to ensure that all dietary components were fully dissolved, and the liquid diet solution was smooth with no lumps. This ensured that the solution was homogenous, and it minimised the possibility of blockages occurring in the feeding nozzle.

2.13.2 Experimental Protocol

Mice were maintained in a reverse lit room to ensure maximal activity during the working day and during experiments, this also meant that feeding would occur during this active phase. In order to ensure enough time was available to make the diet up fresh every day and swapped over with the old diet before the active (dark) phase occurred, a minor alteration to the light:dark cycle was made. Where it had previously been the dark cycle from 8:00AM till 8:00PM, for the purposes of this experiment the dark cycle was shifted by one hour to occur over 9:00AM till 9:00PM. This altered light:dark cycle was initiated two weeks before the administration of the liquid diet to ensure no sudden shift in circadian rhythms in mice involved in the liquid ethanol diet experiment as this could have altered their diet consumption.

The experiment was conducted in two identical halves; this was done purely for logistical reasons due to the time and effort required to adequately maintain and monitor the population size. Each half of the experiment had four cages of mice in groups of four. Two cages were fed control diet and two cages were fed ethanol 5% liquid diet for a period of 14 days. Both cages however received liquid control diet for three days prior to beginning the 14 day ethanol period to habituate mice to the process of feeding on purely liquid diet through a ball bearing operated feed bottle. Water was not given in addition to liquid diet as the diet composition was such that it provided for both nutrient and hydration requirements. The total experimental time is illustrated in Figure 2.5.

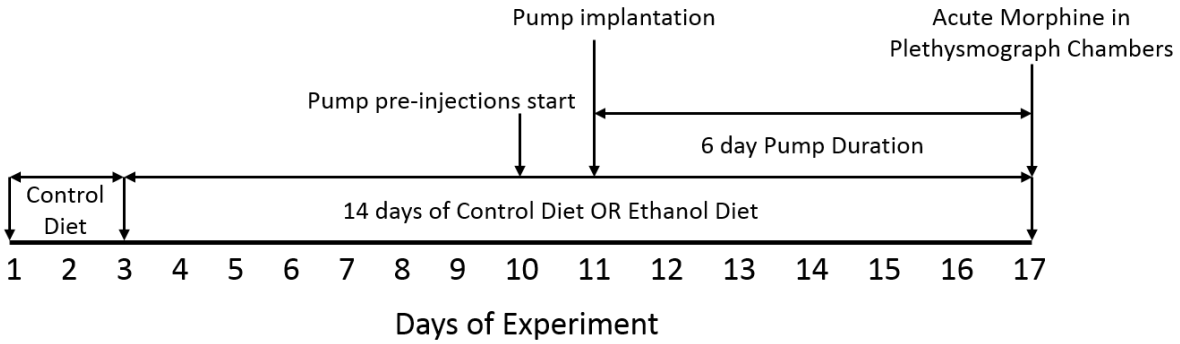


Figure 2.5: Ethanol Diet Protocol*. Timeline schematic of the ethanol diet protocol. Control diet was fed for 3 days to habituate liquid diet feeding. Cages were then fixed to control diet or switched to ethanol (5%) diet. Saline or morphine pre-pump injection began on the morning of the 10th day and a morphine or saline pump was implanted on the evening of the 11th day before an acute morphine challenge on day 17.*

Each bottle of liquid diet was weighed before being inserted in to each cage. Before the addition of fresh diet every morning, each cages bottle of liquid diet was measured so that consumption over the previous day could be recorded. Diet consumption was therefore recorded as a group average for the four mice in each cage. In order to ensure that the liquid diet containing bottles did not become blocked in the ball bearing nozzle, each bottle of liquid diet had its nozzle cleaned four times daily and was inverted multiple times before being placed back in the cage.

2.13.3 Mouse Welfare

The welfare of mice was monitored closely during the liquid diet experiment. This was due to the potentially aversive nature of ethanol which has been demonstrated to occur in mice (Arteel, 2013) as well as the requirement of mice to feed by a novel mechanism, when compared to normal *ad libitum* dried chow and water. Mouse weight was measured prior to administration of control diet for the initial three day period, and then mouse weight was measured every three days thereafter until conclusion of the experiment. Each mouse had its tail individually labelled with permanent marker so that weight could be tracked across each individual mouse. Tail markings were re-applied during every weighing session.

If at any weighing session a mouse showed more than a 20% decrease in body weight when compared to its initial starting weight, then this was considered good reason to humanely cull the mouse such that it did not endure any unnecessary suffering. Mice were also closely monitored for any aggression that may result in submissive mice receiving injuries from alpha males. If a mouse was seen to be consistently enduring aggression, with excessive wounds from other mice within its cage, such that it was suffering unduly, it was also culled due to the reaching of a humane endpoint.

2.13.4 Induction of Tolerance and Measurement of Respiratory Depression

In order to induce tolerance to morphine respiratory depression 3 x 100 mg/kg morphine injections were administered prior to implantation of an as osmotic mini-pump delivering morphine at 45 mg/kg/day for the final 6 days of the diet. See Materials and Methods section 2.4.1.4 for details. Saline pumps were implanted in mice in control and ethanol diet fed cages. Morphine pumps were also implanted in mice in control and ethanol diet fed cages.

Following implantation of osmotic mini-pumps, the respiratory rate of mice in each group was measured each day for 20 min in plethysmography chambers whilst breathing 5% CO_2 in air, as described in Materials and Methods section 2.6. On the final day of experimentation, each mouse had its respiration measured for 20 min, followed by an acute injection of morphine 10 mg/kg i.p..

Respiration was measured for 30 min following this injection window. Following the end of the experiment animals were culled and blood and brain samples taken for measurement of morphine and ethanol content.

2.13.5 Sampling of Mouse Blood and Brain

Samples of both blood and brain were required in order to make quantitative measurement of morphine and ethanol levels following the prolonged ethanol or control diet and implantation of morphine–filled osmotic mini-pumps, followed by an acute morphine injection. Following the end of the experiment, mice were removed from the plethysmograph chambers and placed in opaque carry boxes in pairs.

Each pair of mice were placed within a sealed chamber supplied with 2L/min of pure CO_2 causing an escalation in the chambers CO_2 gas concentration in accordance with Home Office guidelines for the culling of mice via asphyxiation by CO_2. Following death (approximately 2 min after CO_2 exposure), mice were removed from the sealed chambers and placed on their dorsal flank. The ventral abdominal area was incised to expose the gastrointestinal tract of the mouse. The gastrointestinal tract of the mouse was then pushed out of the way using forceps, exposing the descending ventral aorta.

Following exposure of the artery, a 23-gauge, $1^{1/4}$ inch needle attached to a 1 ml syringe (pre-filled with 100µl of 100 units/ml heparin) was inserted into the descending ventral aorta along the length of the artery till the upper bifurcation was reached before blood was removed. This method of sampling allowed for the collection of 800-900µl of blood + heparin, which was then stored in a 1.5 ml Eppendorf tube and frozen at -20^{o}C.

Following the sampling of blood, each mouse was decapitated, and the brain removed intact. Each brain was placed within a small, labelled sealable plastic bag and then placed within a larger sealable plastic bag along with the corresponding animal's blood sample. The bagged samples were then dropped in liquid nitrogen for flash freezing before storing in a -80^{o}C freezer until solvent extraction was performed. The total time from acute morphine injection till flash freezing was no more than 50 min for each mouse.

2.14 Preparation of Plasma and Brain Samples for Morphine Content Analysis

2.14.1 Plasma Sample Preparation

Blood samples were completely defrosted before being spun in a centrifuge for 10 minutes at 10'000 G at a temperature of 4 ^{o}C. Following removal from the centrifuge, 100 μl aliquots of the plasma supernatants were carefully removed and placed into fresh Eppendorfs, ready for extraction.

2.14.2 Brain Sample Preparation

Brains were completely defrosted before being weighed. Each brain was homogenised in phosphate buffer solution (PBS) with 2 ml of PBS added per gram of brain weight. Brain weights varied between 400-500 mg. Following homogenisation, 100 μl aliquots of brain homogenate were carefully removed and placed into fresh Eppendorfs, ready for extraction.

2.14.3 Morphine Extraction from Plasma and Brain Samples

The protocol for preparation of blood and brain samples of mice was kindly provided by the Machlaclan laboratory at Glasgow Caledonian University. In the following section, 'samples' refers to both plasma and brain homogenate samples that were prepared as above. Both sample types were treated identically in the extraction phase.

In addition to the preparation of experimental samples, a calibration curve was prepared to allow correct quantification of morphine levels within experimental samples. A stock solution for the calibration curve was created according to Table 2.3.

Drug	Concentration	Volume Used	Total Volume
Morphine	1 mg/ml	450 μl	n/a
Morphine-3G	1 mg/ml	450 μl	n/a
Hydromorphone	4.5 mg/ml	100 μl	n/a
Solution 1	450 μg/ml for all three drugs	n/a	1000 μl

***Table 2.3. Constitution of stock solution for calibration series**. A 1 ml (1000 μl) stock solution of 450 μg/ml morphine HCl, morphine-3G and hydromorphone was created using the above process.*

In addition to the stock solution and subsequent serial dilutions (Table 4), an internal standard was required within the precipitating solvent, acetonitrile. The internal standard used was deuterated morphine (d3-morphine). To create a stock solution of internal standard solvent, a solution of 1 mg/ml d3-morphine (Sigma Aldrich, UK) was purchased. 100 μl of 1 mg/ml d3-morphine was added to acetonitrile to a total volume of 500 ml. This produced an end concentration of 0.2 μg/ml of d3-morphine. For each standard or experimental solution, 500 μl of this internal standard solvent was used in the precipitation process, an equivalent of 100 μg/ml of d3-morphine.

To produce the calibration curve for sample analysis additional solutions were diluted from stock solution according to the Table 2.4.

Each solution, as defined in Table 4, was used to spike 25 μl into a blank control sample of 100 μl. Following the spiking of the control samples with the calibration curve concentrations of morphine, morphine-3G and hydromorphone, all blank and experimental samples of a volume of 100 μl, had 500 μl of d3-moprhine acetonitrile added to them. This stage precipitated the samples ready for extraction. In addition to the spiked samples in Table 4 and experimental samples two additional samples were prepared. These were a single blank and a double blank sample. The single blank was 100 μl of control sample precipitated with internal standard acetonitrile, whereas the double blank was 100 μl of control sample precipitated with acetonitrile that did not contain d3-morphine.

Following precipitation of all samples with acetonitrile, the solvent was carefully removed and placed into a fresh Eppendorf. Eppendorfs containing solvent and dissolved morphine were then placed in a speedvac with their lids open. Approximately 3 h was required to fully evaporate the entire solvent content of each Eppendorf.

The remaining residue in the bottom of each Eppendorf were then resealed labelled and shipped to the Machlaclan lab at Glasgow Caledonian University for analysis.

Solution	Volume Taken	From Solution	Volume Added Diluent	Concentration µg/ml	Matrix Concentration (25ul spike into 100ul), ng/ml
1	223	Stock	777	100	20000
2	100	1	900	10	2000
3	50	1	950	5	1000
4	25	1	975	2.5	500
5	100	2	900	1	200
6	100	3	900	0.5	100
7	100	4	900	0.25	50
8	100	5	900	0.1	20
9	100	6	900	0.05	10
10	100	7	900	0.025	5
11	100	8	900	0.01	2
12	100	9	900	0.005	1

Table 2.4: Calibration curve concentrations for morphine analysis*. Serial dilution of 100 mg/ml morphine HCl, morphine-3G and hydromorphone and final concentration within 100 ml of mouse plasma to form calibration curve for morphine detection within experimental plasma samples. All volumes expressed in µl.*

2.15 Preparation of Plasma Samples for Ethanol Content Analysis

2.15.1 Plasma Sample Preparation

Blood samples were completely defrosted before being spun in a centrifuge for 10 min at 10'000 G at a temperature of 4 ^{o}C. Following removal from the centrifuge, 100 μl aliquots of the plasma supernatant was careful removed and placed into fresh Eppendorfs.

2.15.2 Plasma Sample Spiking

Based on previous research, plasma levels of ethanol were expected to be approximately 180 mg/dL (Bertola et al., 2013). In order to provide a broader spectrum of known plasma ethanol concentrations with which to compare experimental samples, three different ethanol concentrations within plasma were supplied for analysis. Table 2.5 summarises the dilutions utilised to create these concentrations. Dilution (3) was achieved by adding 10 μl of dilution (2) to 90 μl of control plasma.

Value	Ethanol	Dilution (1)	Dilution (2)	Dilution (3)	Final Concentration
Low	900 mg	1 in 10	1 in 10	1 in 10	90 mg/dL
Expected	900 mg	1 in 10	1 in 5	1 in 10	180 mg/dL
High	900 mg	1 in 10	1 in 2.5	1 in 10	360 mg/dL

Table 2.5. Constitution of known ethanol plasma samples*. Serial dilution of ethanol was performed according to the required end concentration of ethanol within control plasma samples.*

Three plasma samples spiked with each control ethanol concentration were shipped frozen on dry ice, along with all experimental plasma samples to the Machlaclan laboratory at Glasgow Caledonian University for analysis.

2.15.3 Analysis of Plasma and Brain Samples for Morphine Content

Analysis of plasma and brain samples for morphine content was performed by Dr Joanna Roberts in the Machlaclan laboratory at Glasgow Caledonian University. A brief overview of the analysis protocol is described below.

Brain and plasma samples were reconstituted in acetonitrile/H_2O (20/80) and analysed by liquid chromatography (Ultimate 3000 LC system, Dionex, USA)/tandem mass spectrometry (Q-exactive Orbitrap, Thermo-Scientific, USA). Samples were analysed in positive ion mode for morphine, hydromorphone and morphine-3-glucuronide (M-3-G). The quantification range for morphine was between 2.0 and 20,000 ng/ml. Hydromorphone was not found in any of the samples.

2.15.4 Analysis of Plasma Samples for Ethanol Content

Analysis of plasma samples for ethanol content was also performed by Dr Joanna in the Machlaclan laboratory at Glasgow Caledonian University. A brief overview of the analysis protocol is described below.

Ethanol analysis was performed using gas chromatography-mass spectrometry (GCMS) and was carried out on a Thermo Finnigan Trace gas chromatograph with a Thermo Finnigan Voyager GCMS.

The diluent containing the internal standard (1-propanol) was prepared by adding 37 µL of 1-propanol to a 100 ml volumetric flask and making up to the mark with de-ionised water. Standards were prepared by adding 63, 127, 190, 253, 317 and 380 µL of ethanol to separate 100 mL flasks and making up to the mark with de-ionised water to give ethanol concentrations of 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 $mg.dL^{-1}$.

The calibration line was prepared in a headspace vial by mixing 80 µl of each ethanol standard to 1 ml of the diluent solution. The calibration curve had a 1/x fitting applied and all the data was between +/- 15% of the best fit. The samples were prepared by adding 80 µL of sample to 1 mL of the diluent containing the internal standard. The vials were capped, sealed with a crimper and heated at 60°C for 30 min before injected 100 µL of the headspace into the GCMS.

2.16 Experimental Design

2.16.1 Randomisation and Blinding

All treatments were randomly designated to mice. However, mice in one cage always received the same prolonged treatment to decrease the likelihood of aggression between mice receiving two different treatments. Mice were also randomly designated to plethysmograph chambers such that the same treatment did not always provide data from the same plethysmograph chamber.

The experimenter was always blind to either the acute or the prolonged treatment of mice such that the outcome of any given group of mice in an experiment could not be predicted.

2.16.2 Statistical Power Analysis

Prior to the beginning of the experimental investigations that are reported in this thesis, the *in vivo* experimental techniques used in this thesis, primarily respiratory depression and antinociception has been extensively used previously in our laboratory. This provided a large amount of data that could be used as input to a power analysis in order to calculate the correct n number of mice required to ensure experiments were appropriately powered.

All power analyses were carried out using a free downloadable statistical power analysis program (G*Power version 3.1.9).

Prior experimental data produced from previous work was used for these power analyses. This allowed an accurate estimation of expected effect size and thus the power analyses conducted were more likely to predict group sizes of an appropriate size, rather than being under- or overestimated.

Two primary power analyses were conducted. These were conducted to calculate group sizes required for interaction that would result in a minimal and a maximal effect size. This allowed a minimum and maximum group size to be established that should account for all possible interactions.

2.16.2.1 Maximal Effect Size

The greatest effect size was predicted to be the difference between saline injected mice and mice acutely administered an opioid. The difference between 3 means was used to calculate this power and so an – ANOVA: fixed effect omnibus one-way test was used. Group means for the post drug response (expressed as percent of baseline minute volume) to saline and two doses of acute opioid were estimated to be 100, 75 and 50. Standard deviation (S.D.) was set at 0.1 based on previous data with an α-value of 0.05 and a desired power of 0.9.

This produced an estimated effect size (f) of 0.7 with a total n required of 18, meaning 6 animals would be required per group.

2.16.2.2 Minimal Effect Size

Minimal effect size was hypothesized to occur in experiments that were designed to elucidate the interaction between multiple factors. Multiple factors include the presence and absence of prolonged opioid treatment to produce opioid tolerance and the manipulation of developed tolerance with the administration of other drugs.

To assess the interaction of both prolonged opioid treatment and drug manipulations, which represent independent factors, a different type of power analysis was required. To assess power for these experiments an analysis of variance (ANOVA): fixed effect, special, main effects and interactions test was used. An example of a two by two factorial is given in Table 2.6.

Groups/Treatments	Saline	Morphine 10 mg/kg
Non-implanted Mice	X	X/2
Opioid implanted Mice	X	X

Table 2.6. Example of a two by two factorial experiment. *X indicates a given minute volume value with saline in both opioid implanted and non-opioid implanted mice hypothesised to have the same effect and for morphine in opioid implanted mice to have an attenuated effect compared to non-opioid implanted mice. This compares the effect of the opioid implantation and the effect of morphine injection and so a two way analysis of variance (ANOVA) has been used to calculate power with two independent variables present.*

The effect size was estimated to be 0.5, a numerator df of 1 (i.e. levels of variability (2) – 1), an α-value of 0.05 and a desired power of 0.9. This input produced a total n of 36, indicating that an n of 9 per group in a 2 by 2 factorial (as would be the most common set up for analysis in this investigation) would be sufficient to detect statistically significant interactions between multiple factors.

2.17 Data Analysis

Two different primary statistical tests were used during the course of this investigation. These were a One-way ANOVA and a Two-way ANOVA. All data were also subjected to column statistics to test for normality before the appropriate test or post-test was used.

Unless otherwise stated in the figure legend, all data were normally distributed and thus data are given ± standard error of the mean (s.e.m.). All data were analysed using Graphpad Prism version 5.03.

2.17.1 One-way ANOVA

A one-way ANOVA was used to compare multiple groups of mice and their overall responses to different drugs. The only level of variance in these experiments was the drug administered or the dose of drug administered. For normally distributed data Bonferroni's multiple comparisons was utilised. Bonferroni's multiple comparisons allowed the comparison of each dose of drug or each individual drug to be compared to the saline control giving individual p-values for each group.

A repeated measure one-way ANOVA was used to compare a single group that had had a single measure made repeatedly over a period of time that was compared to a baseline value. The variance in these experiments was time.

2.17.2 Two-way ANOVA

A two-way ANOVA was used to compare two or more groups of mice that had two independent variables associated with them. For normally distributed data Bonferroni's multiple comparisons was utilised.

These data are organised into a two by two factorial design as in table 6. The two levels of variance are the ± treatment with an opioid or its absence and the ± presence of an experimental drug. All mice would receive the same challenge dose of opioid, so this was not a variable.

2.17.3 Post Test Choice

Primarily Bonferroni's post-hoc test was used to compare groups. This was chosen over Dunnett's post hoc test. This was due to Bonferroni's post hoc test being acknowledged as a more conservative estimate of statistical significance when compared to Dunnett's (Kim, 2015). Given the power of effect that was explicitly designed within experiments, this should minimise both the occurrence of false positive and false negative results.

3.0 Acute Effects of Opioids

3.1 Introduction

Opioids are a crucial therapy in clinics worldwide for the management of both acute and chronic pain conditions (Benyamin et al., 2008). However, the potency of individual opioids to produce clinically relevant antinociception varies wildly (Vieweg et al., 2005, Drewes et al., 2013). In tandem, the ability of opioid agonists to produce significant, and potentially lethal, respiratory depression varies greatly (Vieweg et al., 2005). Respiratory depression is the major cause of death following opioid overdose (White and Irvine, 1999). This is not restricted to addicted users but is also extremely abundant in therapeutic users who accidentally overdose (Garg et al., 2017).

This eclectic mixture of potency and safety margins requires clinicians and academics to have a solid basis of comparative potency between opioid agonists to work efficiently from. There are clear delineations of any given opioids popularity within different countries of the world (Drewes et al., 2013); understanding the relative potency of opioid agonists allows direct comparisons to be drawn more readily between usage statistics and overdose deaths, despite the use of distinct opioids.

3.1.1 Chapter Aims

The aims of this chapter were:

(i) To characterise the potency of multiple opioids to both depress respiration and induce antinociception, in order to select appropriate doses for further experimentation

(ii) To compare the effect of different opioid receptor selective antagonists on different opioids inducing respiratory depression

3.2 Morphine and Oxycodone

3.2.1 Effect of Morphine on Respiration

Single doses of morphine hydrochloride 1-10 mg/kg (i.p.) administered to mice breathing 95% air + 5% CO_2 produced increasing levels of respiratory depression as doses escalated with both 3 and 10 mg/kg morphine producing significant levels of respiratory depression (Fig. 3.1A). Pre-injection baseline data demonstrates the stabilising effect of the 95% air + 5% CO_2 gas mixture on respiration as well as the general homogeneity of baseline respiration across groups. Saline has no overall effect on mouse respiration (Fig. 3.1A).

Both 3 and 10 mg/kg morphine show significant respiratory depression 5 min after administration and the duration of respiratory depression lasts till the end of the experiment at 35 minutes post-injection. The onset of respiratory depression following morphine administration is rapid, reaching peak effect by 10 min post injection (Fig. 3.1A).

In the event that group baseline data varies slightly, or that there is sizeable variation between individual animal's baseline respiration within a group, each mouse has their post-injection respiration calculated as a percentage of their individual pre-injection baseline. Therefore, the percentage change in respiration for each mouse is presented relative to themselves as a control. This calculation aims to prevents group data being significantly skewed by outlying data points.

In the case of morphine, the percentage baseline data recapitulates the conclusions from the raw data, in that there is a dose dependent decrease in respiration induced by morphine administration and saline administration does not affect mouse respiration (Fig. 3.1B).

Again, the calculated AUC data (Fig. 3.1C) recapitulates the data seen in figures 1a and 1b, demonstrating the dose dependent decrease in respiration followed by morphine administration. This data is ideally suited for both One-way and Two-way ANOVA analysis dependent on the variable(s) within the experiment.

Analysis of respiratory rate and tidal volume as the components of minute volume demonstrate that morphine dose dependently depresses respiration through a decrease in respiratory rate (Fig. 3.2A) with no change seen in tidal volume (Fig. 3.2B).

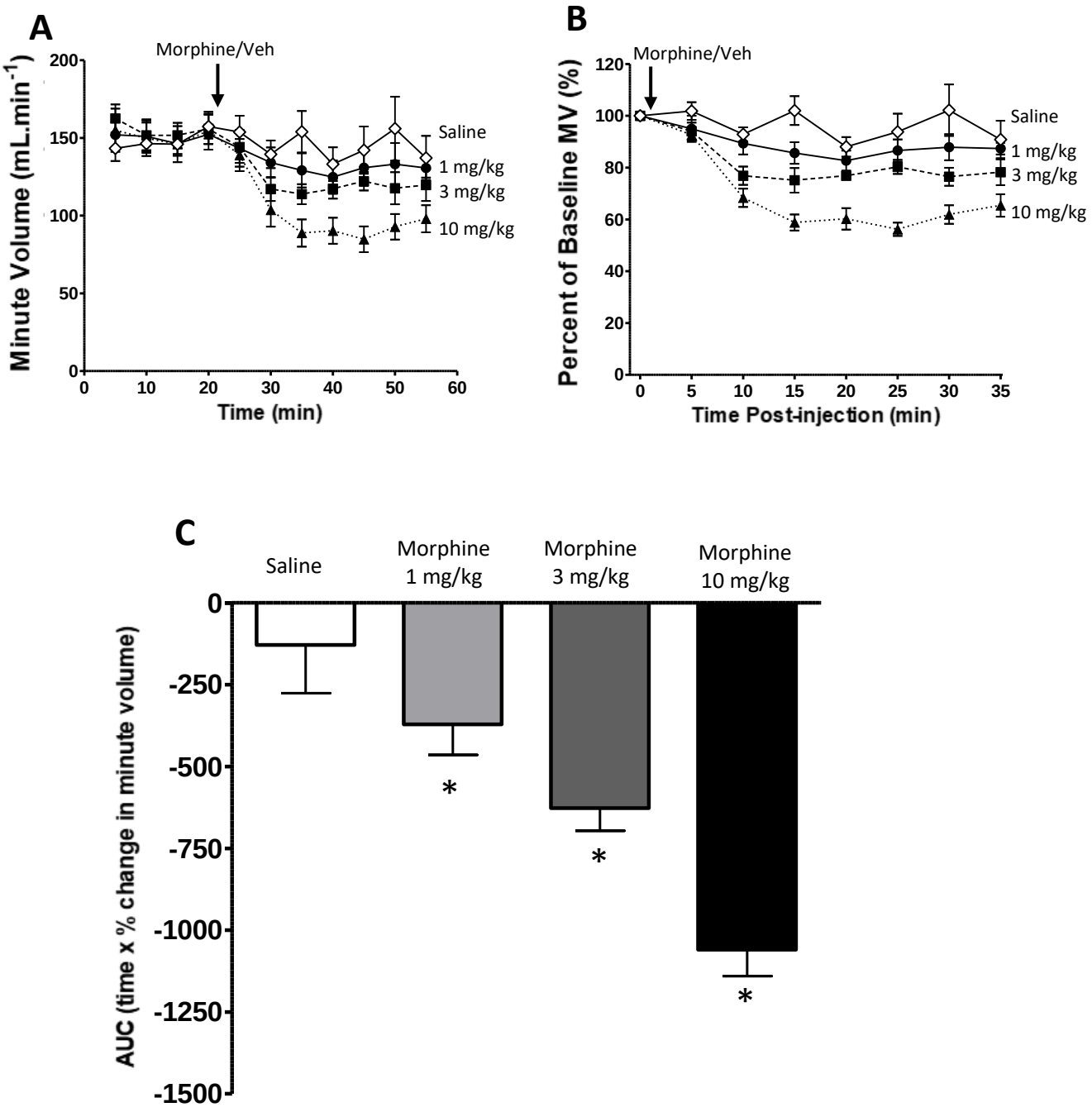


Figure 3.1: Dose dependent depression of minute volume by morphine*. Morphine (1, 3 and 10 mg/kg) administered acutely (i.p) dose dependently depressed the minute volume of CD-1 mice in whole body plethysmographs breathing a 95% air/ 5% CO_2 gas mixture.* ***A****) plots raw minute volume traces with injection indicated by the arrow.* ***B****) plots the percentage decrease in minute volume, calculated for each mouse from individual baseline values.* ***C****) illustrates the overall depression of minute volume calculated by area under the curve (AUC) analysis of the data in (****B****). * indicates significance from saline control where p<0.05. Statistical comparison was made by One-way ANOVA with Bonferroni's comparison made to the saline control group. N=6 for all groups.*

3.2.2 Baseline Respiration and Correlation of Maximum Respiratory Depression

It is possible that mice which exhibit a lower baseline respiration also show a decreased level of opioid induced respiratory depression. Conversely mice that exhibit a higher baseline respiration may show an increased level of opioid induced respiratory depression. This may arise due to a floor effect imposed on opioid respiratory depression. Under such conditions an opioid may not be able to lower mouse respiration below a given threshold. This would skew data derived from mice with lower baselines, as it would appear that the opioid had induced lesser respiratory depression, whereas it would in reality be an artefact of the baseline respiration, not the innate ability of the opioid to depress respiration.

In order to ascertain if this was indeed a possibility, groups of mice that received morphine 10 mg/kg had baseline respiration for the group as well as the individual mouse, plotted against the maximum decrease in respiration seen following administration of morphine. This allowed a linear regression and correlation co-efficient to be calculated.

No correlation between baseline respiration and maximum effect of opioid was observed (Fig. 3.3A & Fig. 3.3B). These data would suggest that the baseline respiration of both individual mice and groups of mice are in no way a predictor of the extent of respiratory depression that will be induced by single opioid dose administration.

3.2.3 Effect of Morphine on Nociception

Morphine 10 mg/kg was chosen as the prototypical challenge dose in the majority of experiments within this thesis. Other opioid challenges were chosen to be equi-potent in terms of the level of respiratory depression induced. Therefore, the antinociceptive effect of morphine was determined only for morphine 10 mg/kg and not other, lower doses.

Significant antinociception was observed 30 minutes after the administration of morphine 10 mg/kg which then appears to plateau until 120 minutes post morphine administration, before a decrease in antinociception is seen at 150 minutes when the experiment is completed (Fig. 3.4). However, mouse tail flick latency is still significantly increased over baseline at the 150-minute time point. Saline on the other hand produced no increase or decrease on the tail flick latency over the whole experiment (Fig. 3.4). This also indicates that the tails were given sufficient time to recover between testing as no sensitisation to testing occurs (i.e. a decrease in tail flick latency) as one might expect if lasting thermal damage were occurring to mouse tails during the experiment.

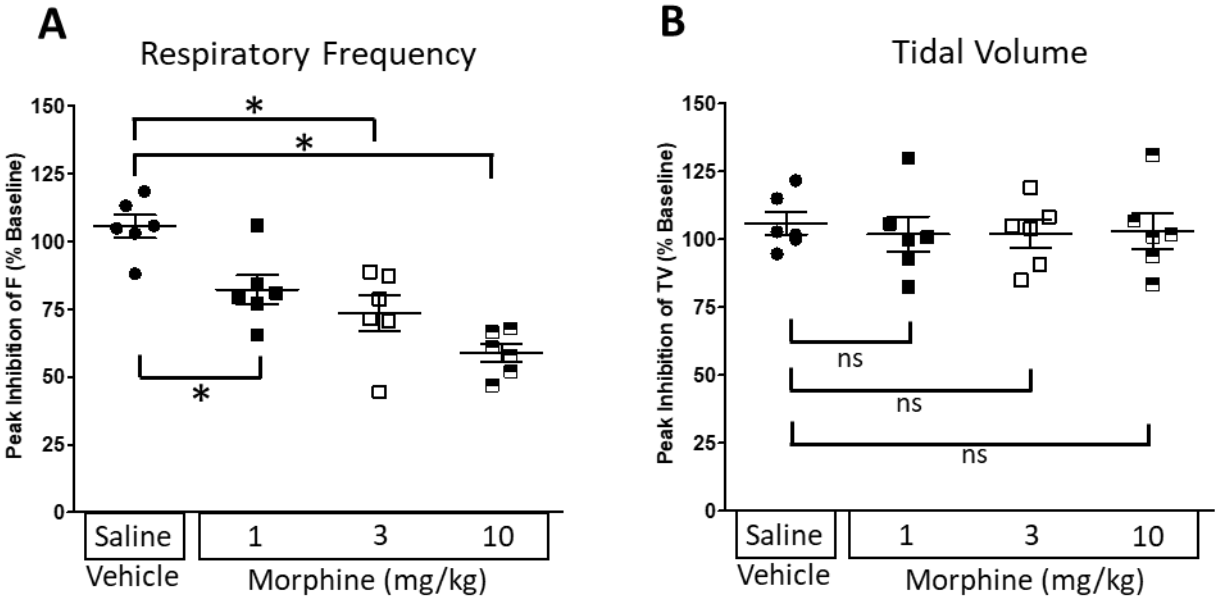


***Figure 3.2: Dose dependent depression of Respiratory Frequency but not Tidal Volume by Morphine**. **A**) Peak inhibition of baseline demonstrates a dose dependent decrease in respiratory frequency by morphine. **B)** Peak inhibition of baseline demonstrates no decrease in tidal volume by morphine. * indicates significance from saline control where p<0.05. Statistical comparison was made by One-way ANOVA with Bonferroni's comparison. N=6 for all groups.*

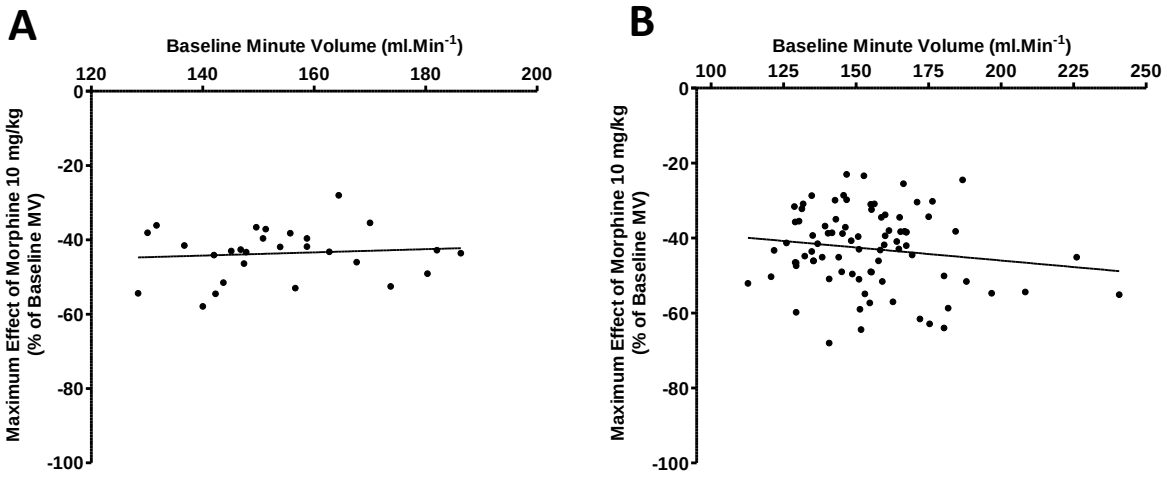


***Figure 3.3: Baseline minute volume does not correlate with the maximum depression of respiration by morphine**. Group minute volume data from pre-morphine baseline was plotted against the maximum group depression of respiration in (**A**). Linear regression was calculated and plotted and the correlation co-efficient between baseline respiration and maximum depression of respiration calculated. In (**A**) group mice were plotted, and in (**B**) individual mice from (**A**) were plotted with baseline vs maximum depression of respiration. The correlation co-efficient for (**A**) is R^2 = 0.009. The correlation co-efficient for (**B**) is R^2 = 0.023. No correlation between baseline minute volume and maximum depression of respiration by opioids can be seen from these data.*

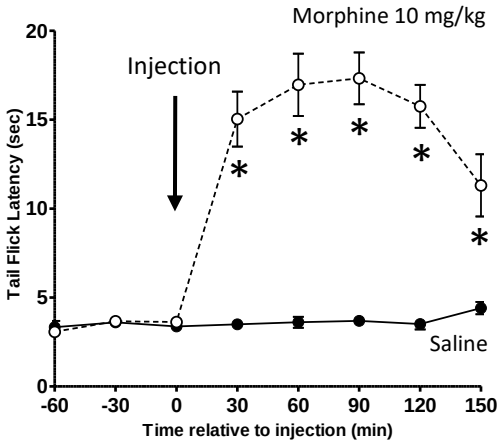


Figure 3.4: Acute morphine produces significant antinociception to a thermal stimulus*. Acute morphine (10 mg/kg) produced a significant produced a significant increase in tail flick latency (30-150 min). Saline however produced no increase or decrease in tail flick latency. Morphine antinociception was maintained for the entire experiment (150 minutes). * indicates significance from pre-injection baseline values where p<0.05. Statistical comparison made by a Repeated Measures One-way ANOVA. N=6 for all groups*

3.2.4 Effect of Oxycodone on Respiration

Oxycodone is an opioid that is currently presenting a critical overdose crisis in the United States of America (USA). Fatal overdoses including oxycodone alone or in combination with other illicit drugs has risen dramatically since the turn of the millennium and represent a significant proportion of opioid overdose death seen in the USA (Kenan et al., 2012, Okie, 2010). Previous data (both clinical and experimental) has indicated that oxycodone is 2-3 times more potent than morphine for antinociception (Ono et al., 2016, Curtis et al., 1999, Jacob et al., 2017) and so doses were adjusted accordingly for these experiments.

Both doses of oxycodone (1 and 3 mg/kg) administered produced rapid and long lasting respiratory depression (Fig. 3.5A & Fig. 3.5B). Similar to that seen with morphine, oxycodone produced significant respiratory depression by 5 minutes and significant respiratory depression was sustained for the entire length of the experiment.

In agreement with previous data in the literature (Jacob et al., 2017) examining the relative potency of oxycodone and morphine to produce tail flick antinociception, these data suggest that oxycodone is approximately three times more potent than morphine for the induction of respiratory depression also.

This can be seen very clearly when comparing the AUC curve values for oxycodone (1 and 3 mg/kg) with morphine (3 and 10 mg/kg) as seen in figure 1c. The saline data presented in Fig. 3.5A-C is the same saline data as presented in figures Fig. 3.1A-C. These experiments were conducted together with the experimenter blind to the treatment. However, for clarity of communication, the morphine and oxycodone data have been presented separately whilst sharing the same saline control group.

Analysis of respiratory rate and tidal volume demonstrates that oxycodone, like morphine, dose dependently depresses respiration through a decrease in respiratory rate (Fig. 3.6A) with no change seen in tidal volume (Fig. 3.6B).

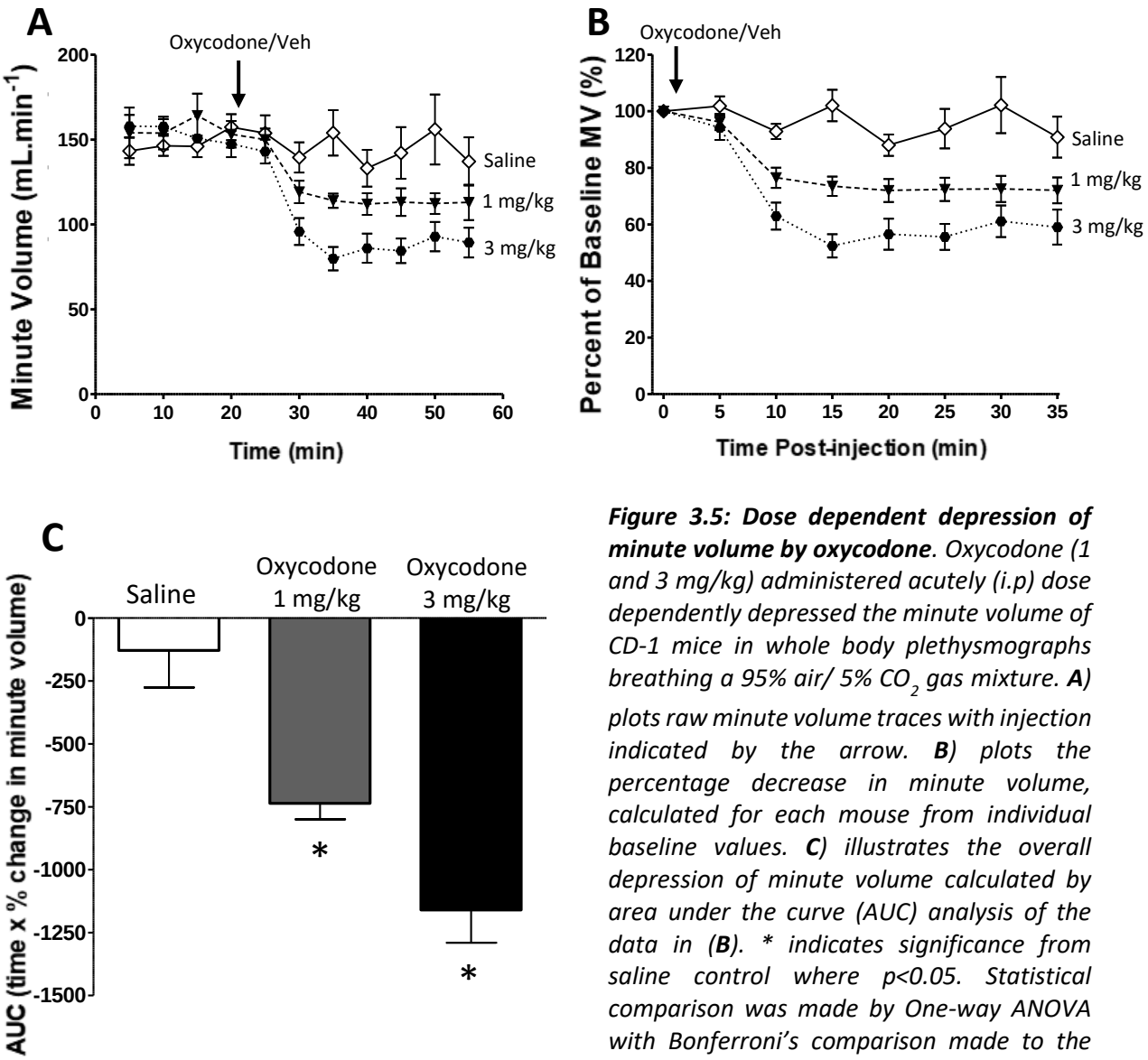


Figure 3.5: Dose dependent depression of minute volume by oxycodone*. Oxycodone (1 and 3 mg/kg) administered acutely (i.p) dose dependently depressed the minute volume of CD-1 mice in whole body plethysmographs breathing a 95% air/ 5% CO_2 gas mixture.* ***A****) plots raw minute volume traces with injection indicated by the arrow.* ***B****) plots the percentage decrease in minute volume, calculated for each mouse from individual baseline values.* ***C****) illustrates the overall depression of minute volume calculated by area under the curve (AUC) analysis of the data in (****B****). * indicates significance from saline control where p<0.05. Statistical comparison was made by One-way ANOVA with Bonferroni's comparison made to the saline control group. N=6 for all groups.*

3.2.5 Effect of Oxycodone on Nociception

Based on both previous literature as well as the previously described respiratory data, both of which that indicated oxycodone has an approximately 3-fold greater potency than morphine, oxycodone 3 mg/kg was thus used in the tail flick assay to assess oxycodone induced antinociception.

Acute administration of oxycodone 3 mg/kg produced a significant antinociceptive effect that peaked at 60 minutes post administration and this peak effect was then sustained for the remainder of the experiment till cessation of testing 150 minutes after oxycodone administration (Fig. 3.7). Similar to previous experimentations (Fig.3.4), there was no increase nor decrease in tail flick latency witnessed with the saline injected group (Fig. 3.7).

These data further validate the relative potency of oxycodone being approximately 3-fold greater than that of morphine.

3.2.6 Receptor Activity of Oxycodone and Morphine

There are three primary opioid receptor sub-types, the μ, κ and δ opioid receptors (MOPr, KOPr and DOPr respectively). Morphine is known to bind almost exclusively to the MOPr whereas there has been some evidence to indicate that oxycodone may bind to both the DOPr (Yang et al., 2016) and the KOPr (Ross and Smith, 1997, Nielsen et al., 2007). To investigate the binding of oxycodone to one or more opioid receptor sub-types *in vivo*, specific antagonists for the DOPr and KOPr were administered prior to challenge with morphine or oxycodone and compared to a pre-treatment with naloxone, the universal opioid antagonist or saline.

3.2.7 Oxycodone Agonist Activity at κ-Opioid Receptors

Nor-Binaltorphimine (Nor-BNI), a specific KOPr antagonist, was administered as a pre-treatment, 24 hours before challenge with either oxycodone or morphine. This length of pre-treatment was established from a solid background literature search that demonstrated 24 hours is required to achieve full blockade of the KOPr (Broadbear et al., 1994, Patkar et al., 2013). Additionally, the dose of Nor-BNI (10 mg/kg) was determined by literature search to achieve a full antagonism of the KOPr. Mice pre-treated with Nor-BNI received an acute injection of oxycodone (3 mg/kg) whilst respiratory parameters were recorded. Fig. 3.8A-C demonstrate that antagonism of the KOPr did not decrease the ability of oxycodone to produce respiratory depression, compared to the full inhibition of oxycodone respiratory depression by a 30-minute pre-treatment with naloxone (1 mg/kg).

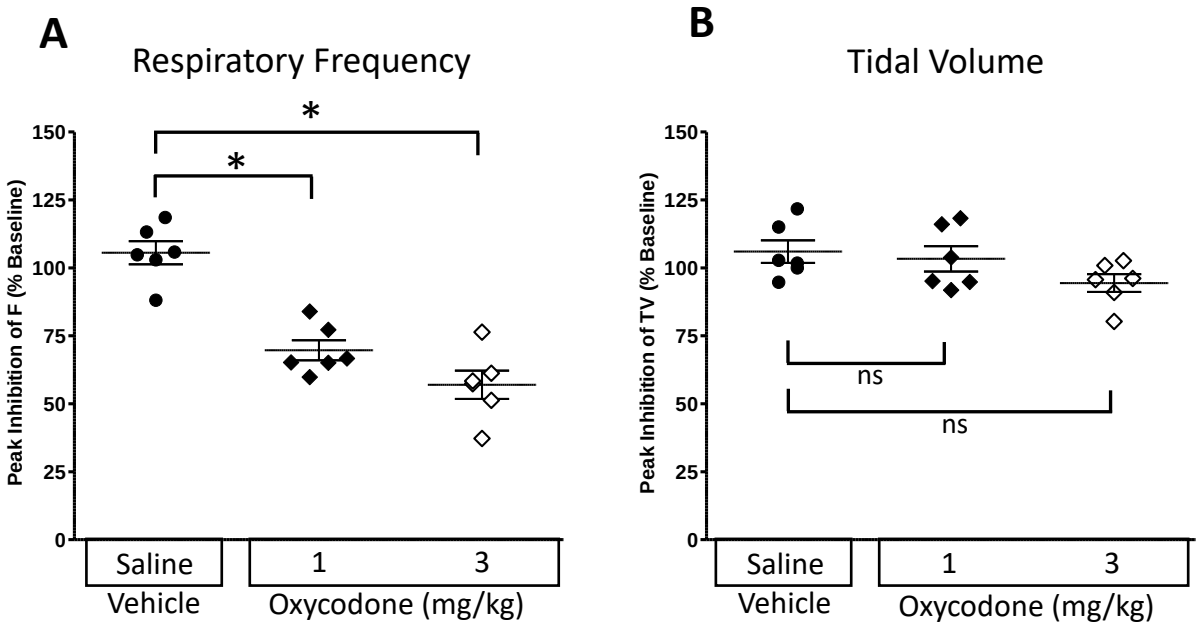


***Figure 3.6: Dose dependent depression of Respiratory Frequency but not Tidal Volume by Oxycodone**. **A**) Peak inhibition of baseline demonstrates a dose dependent decrease in respiratory frequency by oxycodone. **B)** Peak inhibition of baseline demonstrates no decrease in tidal volume by oxycodone. * indicates significance from saline control where p<0.05. Statistical comparison was made by One-way ANOVA with Bonferroni's comparison. N=6 for all groups.*

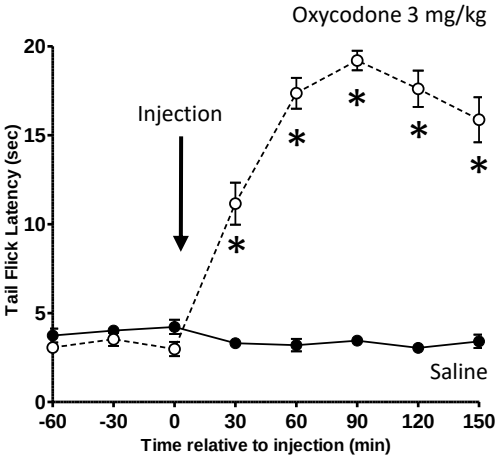


***Figure 3.7: Acute oxycodone produces significant antinociception to a thermal stimulus**. Acute oxycodone (3 mg/kg) produced a significant increase in tail flick latency (30-150 min). Saline however produced no increase or decrease in tail flick latency. Oxycodone antinociception was maintained for the entire experiment (150 minutes). * indicates significance from pre-injection baseline values where p<0.05. Statistical comparison made by a Repeated Measures One-way ANOVA. N=6 for all groups.*

3.2.8 Oxycodone Agonist Activity at δ-Opioid Receptors

There is published literature indicating that oxycodone may have agonist activity at the DOPr (Yang et al., 2016). Naltrindole (10 mg/kg) was administered as a specific DOPr antagonist 30-minutes prior to acute administration of morphine (10 mg/kg) or oxycodone (3 mg/kg). Fig. 3.8A-C indicate that whilst DOPr inhibition appeared to decrease the extent of oxycodone induced respiratory depression, over the course of 30 minutes this did not reach statistical significance.

3.2.9 Nor-BNI Antagonism of the Specific κ-opioid Agonist U69,593

However, due to the lack of antagonist activity on morphine and oxycodone induced respiratory depression, an additional control group was performed to determine that our dose and pre-treatment paradigm with Nor-BNI had indeed provided full antagonist block of the KOPr. U69,593 is a highly selective KOPr agonist that has been reported to have some antinociceptive potency in mice (Horan et al., 1992, Patkar et al., 2013, Kuo et al., 2015). A dose of U69,593 (20 mg/kg) twice that reported to provide antinociception in mice (Patkar et al., 2013)was acutely administered (i.p.) to mice that had either received a saline injection or Nor-BNI injection 24 hours previously. Prior to and following U69,593 administration, the tail flick latency of mice was measured to assess the antinociceptive effect of U69,593 plus or minus Nor-BNI.

Fig. 3.9 shows that U69,593 provides a slight antinociceptive response in mice pre-treated with saline. This antinociception is completely inhibited in mice pre-treated with Nor-BNI (10 mg/kg) (Fig. 3.9). This validates the Nor-BNI paradigm chosen to effect complete antagonism of the KOPr receptor and recapitulates the conclusion that oxycodone does not show KOPr agonism *in vivo*, at least in relation to oxycodone induced respiratory depression.

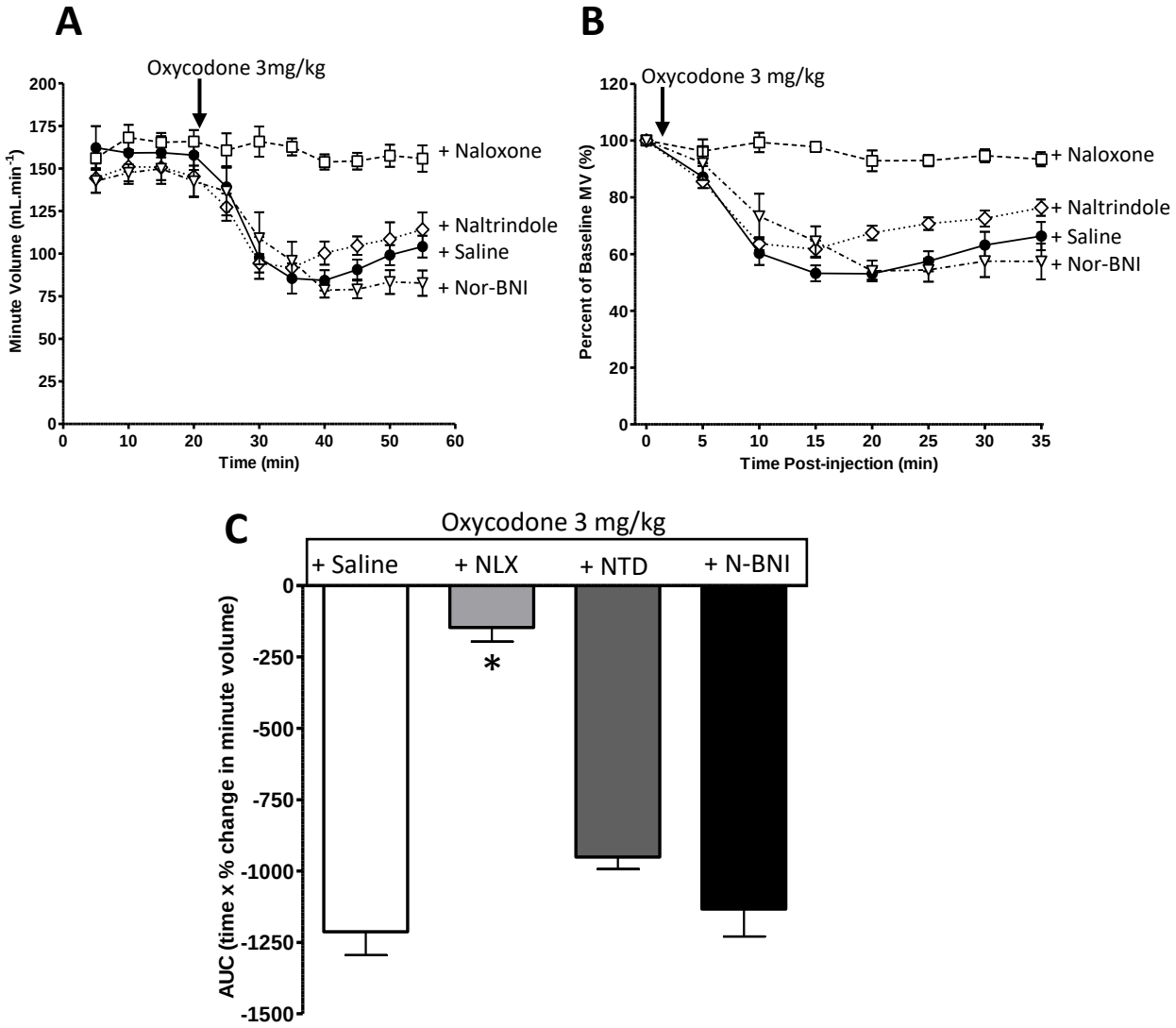


Figure 3.8: Antagonism of KOPr by Nor-BNI or DOPr by naltrindole does not alter oxycodone induced respiratory depression*. Saline administration 30mins prior to administration of oxycodone (3 mg/kg) did not alter the degree of oxycodone induced respiratory depression (**A, B and C**). 30min pre-treatment with naltrindole (10 mg/kg) or 24 hour pre-treatment with nor-BNI (10 mg/kg) also did not significantly alter the degree of respiratory depression induced by oxycodone (**A, B and C**). * indicates significance from saline control where p<0.05. Statistical comparison was made by One-way ANOVA with Bonferroni's comparison made to the saline control group. N=6 for all groups. All drugs/vehicle administered i.p.*

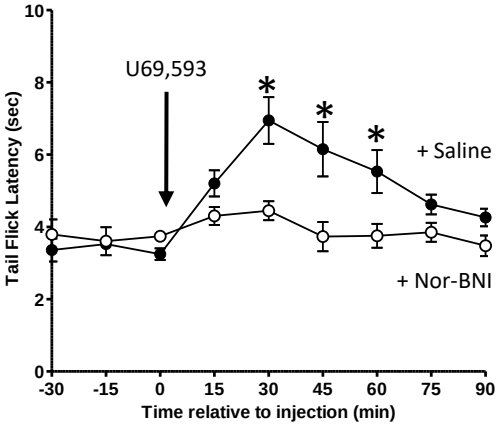


Figure 3.9: Nor-BNI provides complete block of U69,593 mediated antinociception*. The KOPr agonist U69,593 (20 mg/kg) produced a small but significant increase in tail flick latency (30-60 min). However, 24hr pre-treatment with Nor-BNI a specific KOPr antagonist completed inhibited U69,593 mediated antinociception. * indicates significance from pre-injection baseline values where p<0.05. Statistical comparison made by a Repeated Measures One-way ANOVA. N=6 for all groups.*

3.2.10 Morphine Agonist Activity at δ– / κ-Opioid Receptors

Using an identical method for administering naloxone, naltrindole and nor-BNI, the receptor selective agonist activity of morphine was investigated as a positive control. Morphine acts as a positive control as it is well reported to be a largely specific agonist for the MOPr alone (Loh et al., 1998, Matthes et al., 1996). Naloxone pre-treatment completely inhibited morphine respiratory depression, however neither naltrindole or nor-BNI pre-treatment affected any change in morphine respiratory depression when compared to saline pre-treated controls (Fig. 3.10A-C).

From these data, we can conclude that morphine respiratory depression is mediated exclusively by the MOPr with neither DOPr or KOPr antagonism inhibiting morphine induced respiratory depression. Similarly, KOPr antagonism did not inhibit oxycodone induced respiratory depression. DOPr antagonism however, did appear to present a trend towards inhibiting oxycodone induced respiratory depression, though this did not reach statistical significance. An increased dose of naltrindole was not used, as at higher concentrations naltrindole does bind to the MOPr (Raynor et al., 1994, Rogers et al., 1990), thus if a higher dose further inhibited oxycodone induced respiratory depression, this may be due to antagonism of both the MOPr and DOPr. This would prevent solid conclusions being drawn on the receptor agonist activity of oxycodone.

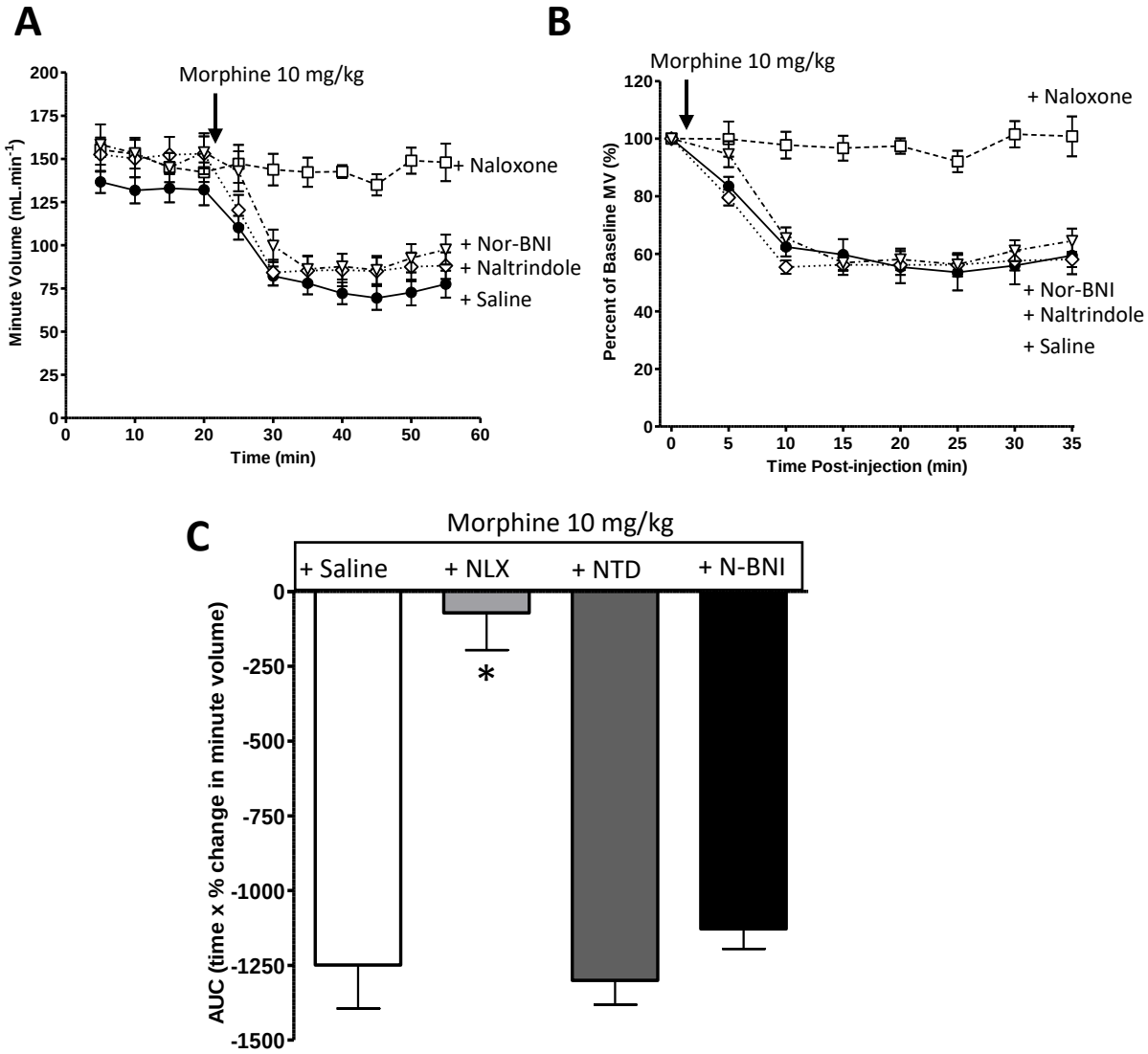


Figure 3.10: Antagonism of KOPr by Nor-BNI or DOPr by naltrindole does not alter morphine induced respiratory depression. *Saline administration 30mins prior to administration of morphine (10 mg/kg) did not alter the degree of morphine induced respiratory depression (**A, B and C**). 30min pre-treatment with naltrindole (10 mg/kg) or 24 hour pre-treatment with nor-BNI (10 mg/kg) also did not significantly alter the degree of respiratory depression induced by morphine (**A, B and C**). * indicates significance from saline control where p<0.05. Statistical comparison was made by One-way ANOVA with Bonferroni's comparison made to the saline control group. N=6 for all groups. All drugs/vehicle administered i.p.*

3.3 Methadone

3.3.1 Effect of Methadone on Respiration

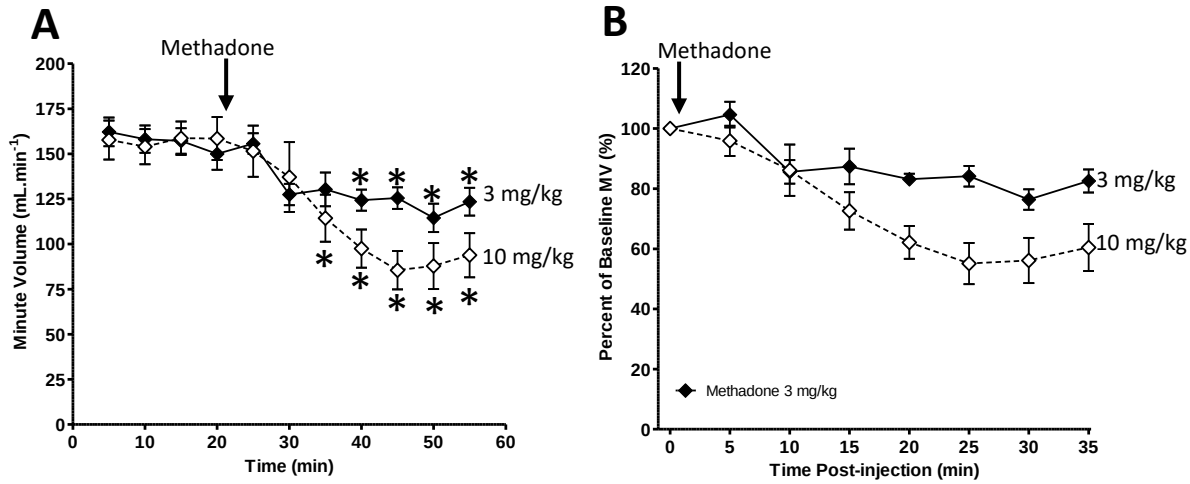
Methadone is unique amongst the opioid used experimentally within this thesis. Unlike the other opioids that are used primarily as clinical analgesics, methadone finds far greater use as a substitution opioid in the long-term treatment of opioid addiction (Darke and Ross, 2001, Darke et al., 2003). That is not to say that methadone does not have the potential for both abuse and lethal overdose (Darke and Ross, 2001). Methadone is considered to have a lower abuse liability than other conventional opioids. Additionally methadone is thought to have good clinical efficacy at preventing respiratory depression that might occur following "on top" use of opioids by an addict in search of euphoria whilst undergoing substitution therapy (Kreek et al., 2010, Cornish et al., 2010).

Previous data within the lab has indicated that methadone has a similar potency to that of morphine. As such methadone was administered at only two doses, 3 and 10 mg/kg, to parallel the two highest doses of morphine administered.

Methadone was found, as morphine and oxycodone, to produce rapid and prolonged respiratory depression, with an onset of significant respiratory depression seen within the first 5 minutes following administration and a depression of respiration that lasted the duration of the experiment till recording ceased (Fig. 3.11A-B).

AUC analysis of methadone depression of respiration seen in Fig. 3.11B demonstrates that the overall level of respiration depression induced by methadone 10 mg/kg (Fig. 3.11C) is approximately -1000 AUC. This is of a similar value (approx. -1000) to that seen with morphine 10 mg/kg (Fig 3.1C). Methadone is often cited as having both a higher efficacy and potency than morphine in multiple *in vitro* experiments (Selley et al., 1998, McPherson et al., 2010, Saidak et al., 2006, Borgland et al., 2003) and so this result is somewhat surprising. One might predict that methadone would also be more potent than morphine *in vivo*. Indeed, methadone has been shown to produce more potent antinociception than morphine in rats for several assays, including thermal, mechanical and neuropathic pain states (Lemberg et al., 2006), as well as for the alleviation of pain depressed behaviour (Altarifi et al., 2015).

Analysis of respiratory rate and tidal volume demonstrates that methadone, like oxycodone and morphine, dose dependently depresses respiration through a decrease in respiratory rate (Fig. 3.12A) with no change seen in tidal volume (Fig. 3.12B).



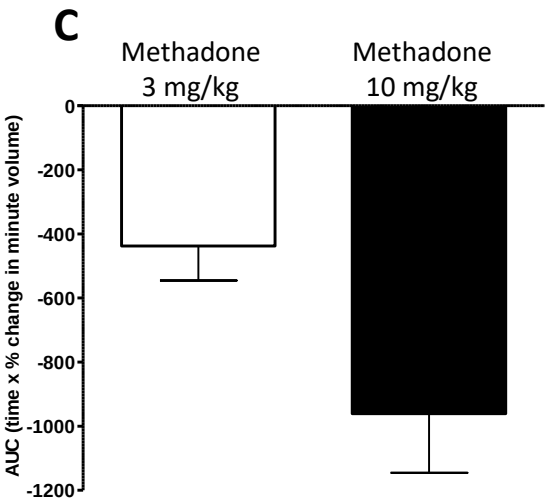


Figure 3.11: Dose dependent depression of minute volume by methadone*. Methadone (3 and 10 mg/kg) administered acutely (i.p) dose dependently depressed the minute volume of CD-1 mice in whole body plethysmographs breathing a 95% air/ 5% CO_2 gas mixture.* ***A****) plots raw minute volume traces with injection indicated by the arrow.* ***B****) plots the percentage decrease in minute volume, calculated for each mouse from individual baseline values.* ***C****) illustrates the overall depression of minute volume calculated by area under the curve (AUC) analysis of the data in (****B****). * indicates significance from baseline where p<0.05. Statistical comparison was made by Repeated measures Two-way ANOVA with Bonferroni's comparison. N=6 for all groups.*

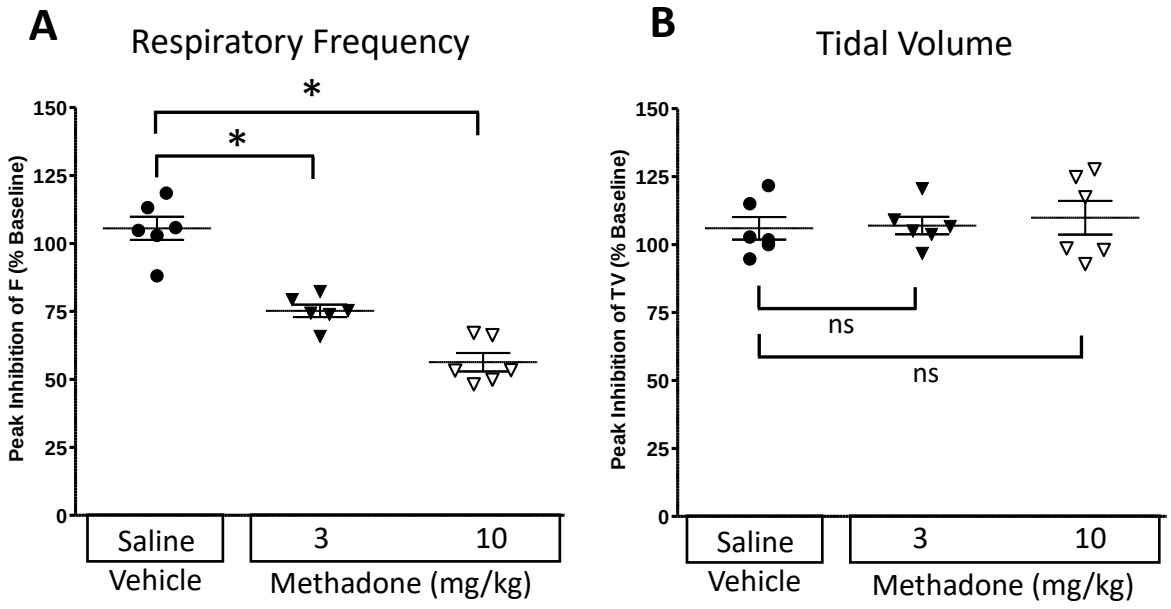


***Figure 3.12: Dose dependent depression of Respiratory Frequency but not Tidal Volume by Methadone**. **A**) Peak inhibition of baseline demonstrates a dose dependent decrease in respiratory frequency by methadone. **B)** Peak inhibition of baseline demonstrates no decrease in tidal volume by methadone. * indicates significance from saline control where p<0.05. Statistical comparison was made by One-way ANOVA with Bonferroni's comparison. N=6 for all groups.*

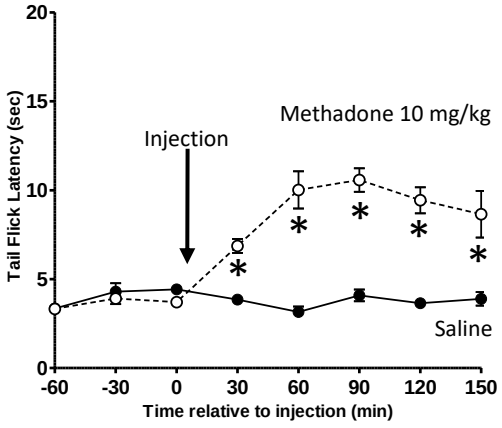


***Figure 3.13: Acute methadone produces significant antinociception to a thermal stimulus.** Acute methadone (10 mg/kg) produced a significant increase in tail flick latency (30-150 min). Saline produced no increase or decrease in tail flick latency. Methadone antinociception was maintained for the entire experiment (150 minutes). Statistical comparison made by a Repeated Measures Two-way ANOVA. * indicates significance from pre-injection baseline values where p<0.05. N=6 for all groups.*

3.3.2 Effect of Methadone on Nociception

Acute administration of methadone 10 mg/kg produced a significant increase in tail flick latency 30 minutes' post administration. The level of antinociception seen by 30 minutes was maintained as the maximum level of antinociception till cessation of the experiment at 150 minutes' post methadone administration (Fig. 3.13).

These data suggest that whilst methadone is as potent as morphine when producing respiratory depression in mice, it appears to have a decreased potency in producing an antinociceptive effect in the warm water tail flick in mice. Methadone produced prolonged antinociception (Fig. 3.13) but had a noticeably reduced maximum effect on tail flick latency enhancement in the context of morphine (Fig. 3.4).

Mitigating factors in this conclusion include the time difference in performing the methadone and morphine. These experiments were not completed in tandem and it is possible that had a morphine group been included alongside the methadone, the morphine would also have shown a decreased maximum. This may have occurred due to either an artefact presented in the batch of mice used, or indeed some experimenter error in handling and/or dosing.

Additionally, methadone is thought to be rapidly metabolised in the mouse compared to morphine (Kalvass et al., 2007a). However, one would consider that this metabolism would equally effect both central and spinally processed pathways such as respiratory control and nociceptive reflexes respectively. Alternatively, a difference in receptor density at the central and spinal level may account for this difference.

3.4 Fentanyl

3.4.1 Effect of Fentanyl on Respiration

Fentanyl is an extremely potent opioid agonist with a plethora of clinical uses including pain management as well as use within anaesthesia. However, in recent years, particularly within the USA, there has been a surge in abuse of fentanyl as well as its even more potent analogue carfentanil (Marshall et al., 2017, Lee et al., 2016). Due to the extreme potency of fentanyl, there is a much smaller safety tolerance when it comes to dosing, as slight increases in material weight can shift dosing from safe to lethal. As such, not only are the number of addicts abusing fentanyl on the rise, but indeed the number of fatal overdoses involving fentanyl are on the rise. Fentanyl abuse and lethal overdose by fentanyl, represents the latest step of an escalating opioid epidemic within the USA that is pushing care facilities and law enforcement to breaking point.

Previous publications that have utilised fentanyl for antinociception as well as respiratory depression were used to guide initial dose choices of fentanyl to be used in studying depression of respiration by fentanyl (Sahbaie et al., 2006, Melief et al., 2010). Doses of 0.05 and 0.15 mg/kg were decided on as initial doses with the hypothesis that these would produce similar levels of respiratory depression to 3 and 10 mg/kg morphine.

Both doses of fentanyl rapidly depressed mouse respiration and the extent of respiratory depression was maintained for the duration of measurement (Fig 3.14A-B). The level of respiratory depression induced by 0.15 mg/kg fentanyl displayed in Fig. 3.14B-C is analogous to that of morphine (10 mg/kg) as demonstrated previously (Fig. 3.1B-C) and thus was determined to be the best dose for comparison with morphine.

Analysis of respiratory rate and tidal volume demonstrates that fentanyl dose dependently depresses respiration through a decrease in respiratory rate (Fig. 3.15A). However, unlike methadone, oxycodone and morphine; at the highest examined dose, fentanyl also produces a significant depression in tidal volume (Fig. 3.15B).

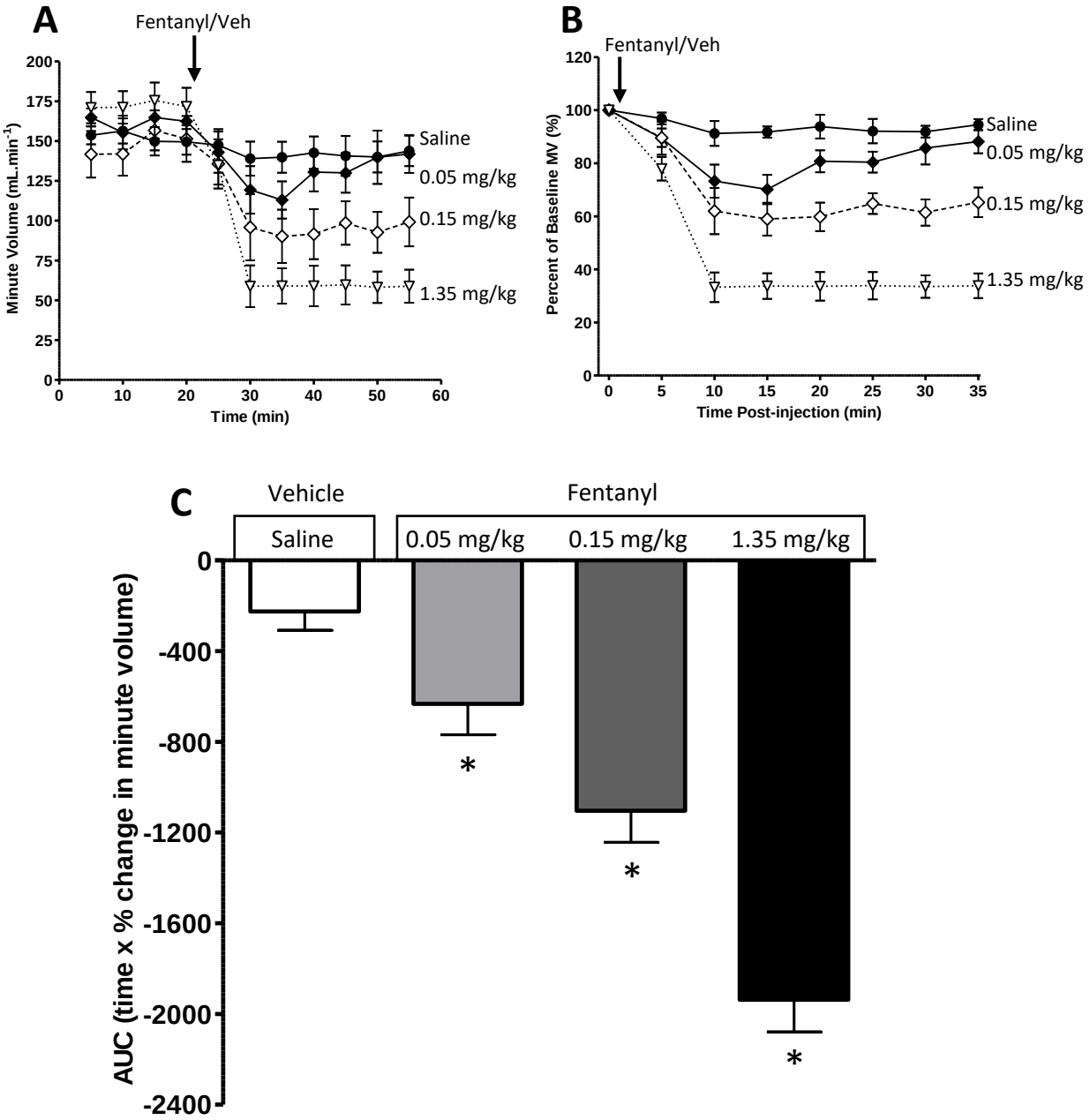


Figure 3.14: Dose dependent depression of minute volume by fentanyl*. Fentanyl (0.05 and 0.15 mg/kg) administered acutely (i.p) dose dependently depressed the minute volume of CD-1 mice in whole body plethysmographs breathing a 95% air/ 5% CO_2 gas mixture.* ***A****) plots raw minute volume traces with injection indicated by the arrow.* ***B****) plots the percentage decrease in minute volume, calculated for each mouse from individual baseline values.* ***C****) illustrates the overall depression of minute volume calculated by area under the curve (AUC) analysis of the data in (****B****). * indicates significance from saline control where p<0.05. Statistical comparison was made by One-way ANOVA with Bonferroni's comparison made to the saline control group. N=6 for all groups.*

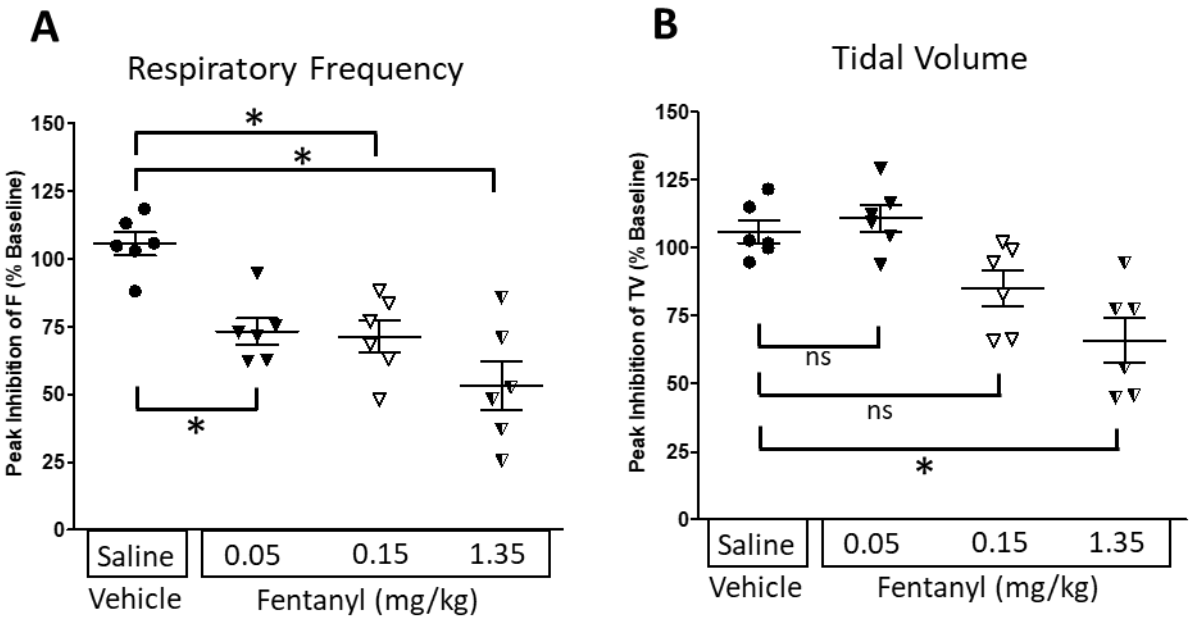


***Figure 3.15: Dose dependent depression of Respiratory Frequency and Tidal Volume by Fentanyl**. **A**) Peak inhibition of baseline demonstrates a dose dependent decrease in respiratory frequency by fentanyl **B)** Peak inhibition of baseline demonstrates a dose dependent decrease in tidal volume by fentanyl. * indicates significance from saline control where p<0.05. Statistical comparison was made by One-way ANOVA with Bonferroni's comparison. N=6 for all groups.*

3.4.2 Naloxone Reversal of Fentanyl Respiratory Depression

The recent fentanyl epidemic has result in an increase in clinical observation regarding fentanyl overdose situations. One observation arising is that fentanyl requires significantly greater amounts of naloxone administration to antagonise fentanyl induced respiration depression, compared to the amount of naloxone required to antagonise respiratory depression induced by heroin (CDC, 2015, CDC, 2016).

To investigate this potential phenomenon, doses of morphine (10 mg/kg) and fentanyl (0.15 mg/kg) that are equi-potent at inducing respiratory depression were administered and compared to control saline injected mice. In all three experiments conducted, morphine and fentanyl produced the same percentage decrease in baseline minute volume as each other (Fig. 3.16B, Fig. 3.17B & Fig. 3.18B), demonstrating the equi-potent nature of these doses with regards to depressing mouse minute volume. Differences in baseline minute volume were seen between morphine administered mice and saline or fentanyl administered mice in two of the three experiments (Fig. 3.16A & Fig. 3.17A), however, as mice were randomly designated to experimental groups and as the percentage decrease was not different in these mice then this is not considered to confound the experiment.

Following 20 min of opioid exposure in each experiment, which allowed peak depression of minute volume by morphine and fentanyl to occur, a dose of naloxone was administered at either 0.3, 1 or 3 mg/kg. All three doses of naloxone provided sufficient antagonism to reverse morphine depression of minute volume (Fig. 3.16C, Fig. 3.17C & Fig. 3.18C), however only administration of naloxone 3 mg/kg was able to fully antagonise and reverse fentanyl depression of minute volume (Fig. 3.18C).

These data suggest that for some unknown reason, fentanyl depression of respiration may be significantly harder to antagonise through naloxone administration than respiratory depression induced by heroin. This has obvious significance in the immediate requirement of determining the opioid used in an overdose situation and the requisite amount of naloxone then required to provided recovery of respiratory function.

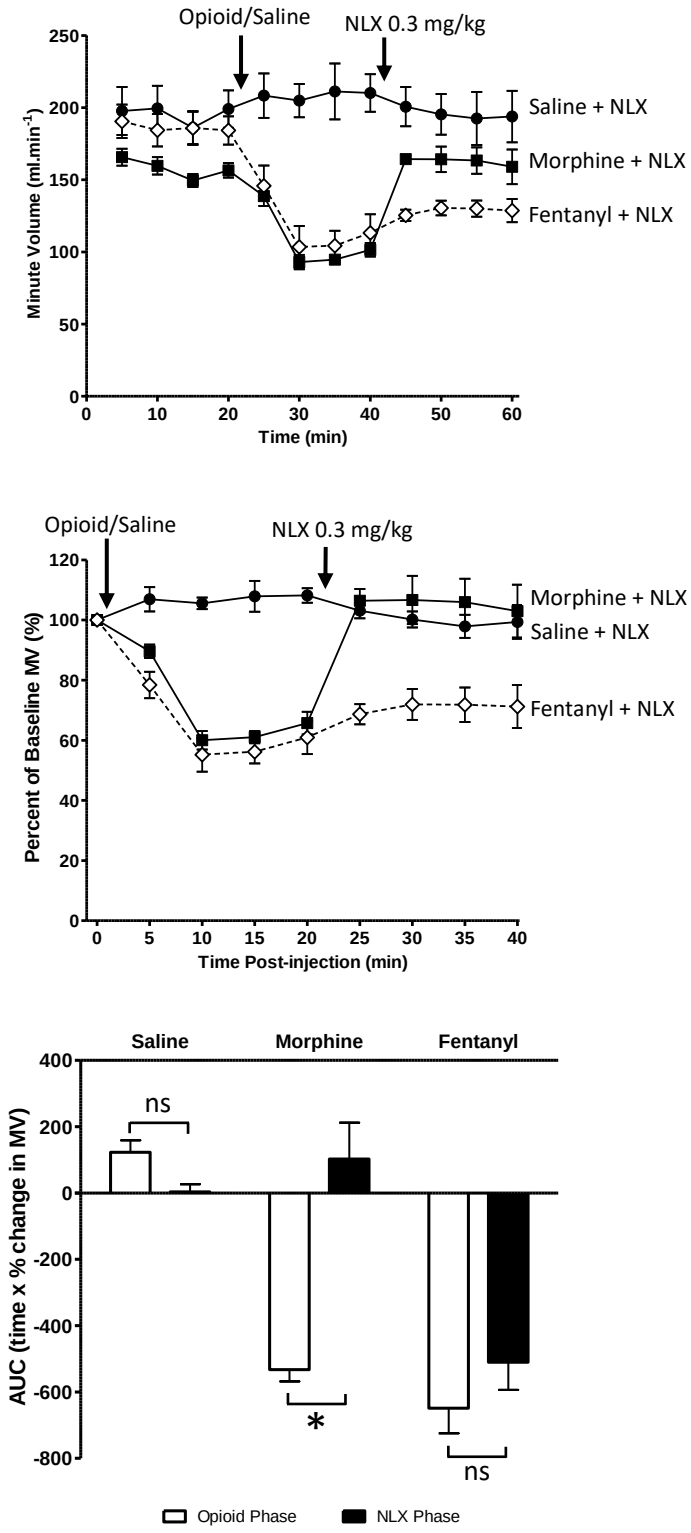


***Figure 3.16: Reversal of Morphine and Fentanyl Respiratory Depression by Naloxone 0.3 mg/kg**. **A-B**) Acute injections (i.p.) of morphine (10 mg/kg) and fentanyl (0.15 mg/kg) were equipotent at depressing minute volume compared to saline control. 20 Min after opioid/saline injection, naloxone (NLX 0.3 mg/kg i.p.) was administered which completely reversed morphine depression of minute volume but did not effect fentanyl depression of minute volume. Saline injected minute volume was unaffected. **C**) Area under the curve (AUC) was measured from (**B**) for 0-20 min and 25-40 min to compare the degree of minute volume depression before (Opioid Phase) and after (NLX Phase) naloxone administration. Significant reversal of morphine but not fentanyl depression of minute volume was seen.* indicates significance from opioid phase control where p<0.05. Statistical comparison was made a Two-way Paired Student's T-test. N=6 for all groups.*

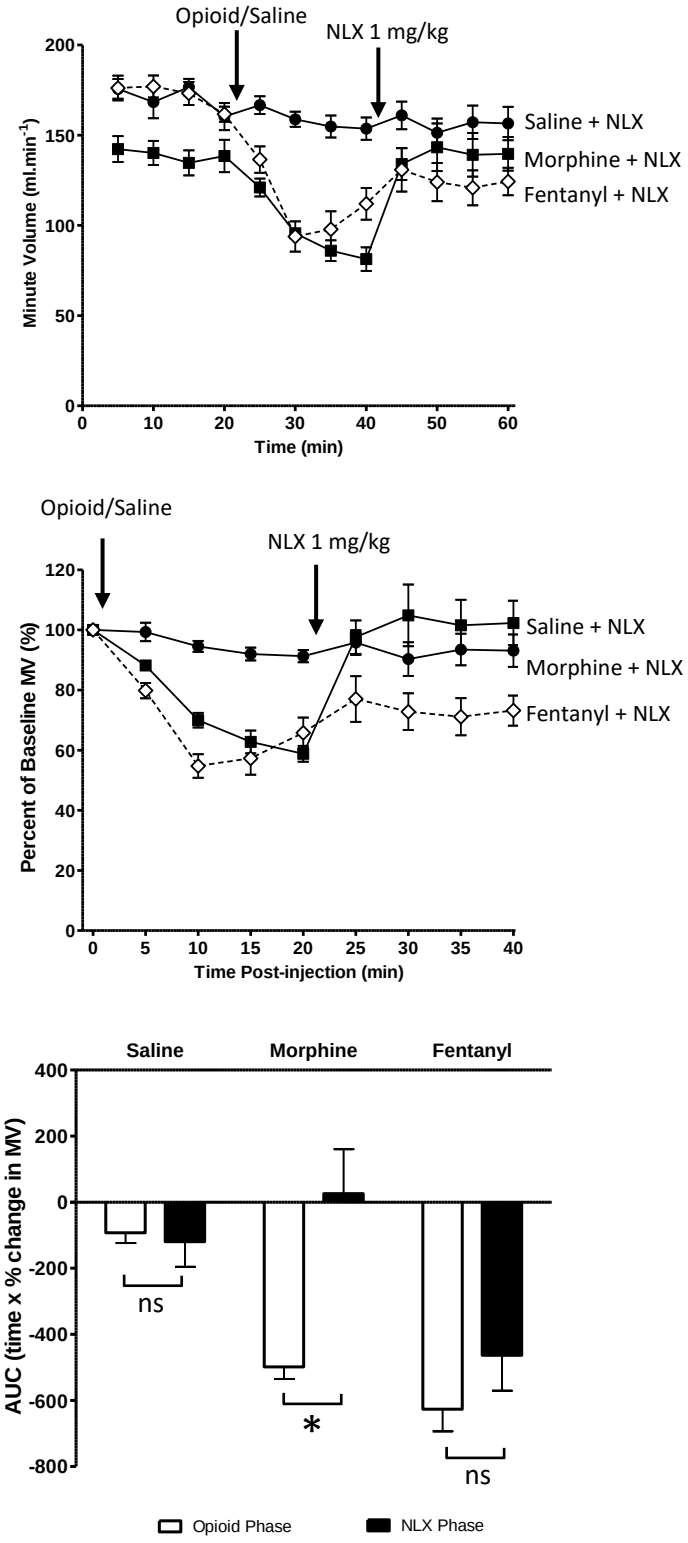


Figure 3.17: Reversal of Morphine and Fentanyl Respiratory Depression by Naloxone 1 mg/kg. ***A-B****) Acute injections (i.p.) of morphine (10 mg/kg) and fentanyl (0.15 mg/kg) were equipotent at depressing minute volume compared to saline control. 20 Min after opioid/saline injection, naloxone (NLX 1 mg/kg i.p.) was administered which completely reversed morphine depression of minute volume but did not effect fentanyl depression of minute volume. Saline injected minute volume was unaffected.* ***C****) Area under the curve (AUC) was measured from (****B****) for 0-20 min and 25-40 min to compare the degree of minute volume depression before (Opioid Phase) and after (NLX Phase) naloxone administration. Significant reversal of morphine but not fentanyl depression of minute volume was seen.* indicates significance from opioid phase control where p<0.05. Statistical comparison was made a Two-way Paired Student's T-test. N=6 for all groups.*

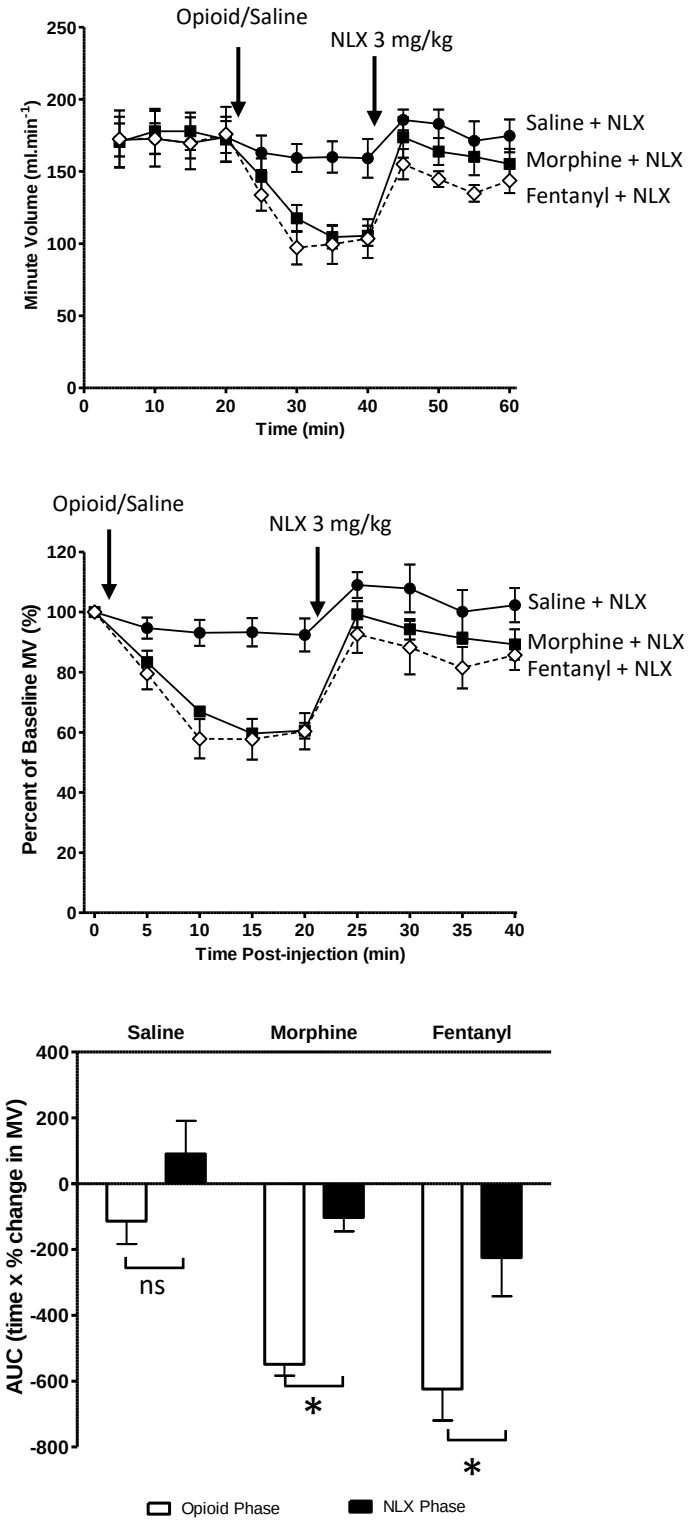


Figure 3.18: Reversal of Morphine and Fentanyl Respiratory Depression by Naloxone 3 mg/kg*. **A-B**) Acute injections (i.p.) of morphine (10 mg/kg) and fentanyl (0.15 mg/kg) were equipotent at depressing minute volume compared to saline control. 20 Min after opioid/saline injection, naloxone (NLX 3 mg/kg i.p.) was which completely reversed morphine depression of minute volume and fentanyl depression of minute volume. Saline injected minute volume was unaffected. **C**) Area under the curve (AUC) was measured from (**B**) for 0-20 min and 25-40 min to compare the degree of minute volume depression before (Opioid Phase) and after (NLX Phase) naloxone administration. Significant reversal of morphine and fentanyl depression of minute volume was seen.* indicates significance from opioid phase control where p<0.05. Statistical comparison was made a Two-way Paired Student's T-test. N=6 for all groups.*

3.4.3 Effect of Fentanyl on Nociception

Acute fentanyl (0.15 mg/kg) produced a significant increase in the tail flick latency of mice 30 minutes following administration (Fig. 12). However, unlike the previously tested opioids of morphine, oxycodone and methadone, fentanyl antinociception was not maintained for the duration of testing. Following peak antinociceptive effect at 30 minutes' post administration, fentanyl antinociception decreased every 30 minutes upon measurement until returning to baseline levels at 120 minutes' post administration (Fig. 3.19).

The data derived from fentanyl antinociception leads us to the conclusion that if a prolonged measurement of fentanyl respiratory depression were made, then this would also show a relatively short duration of action when compared to the other experimental opioids. This decreased duration of action has important utility in the experimental investigation of fentanyl. Due to its relatively rapid clearance, or development of tolerance, fentanyl may be administered repeatedly over much shorter time frames. This is a significant difference compared to the lasting duration of oxycodone, morphine and methadone in the tail flick assay.

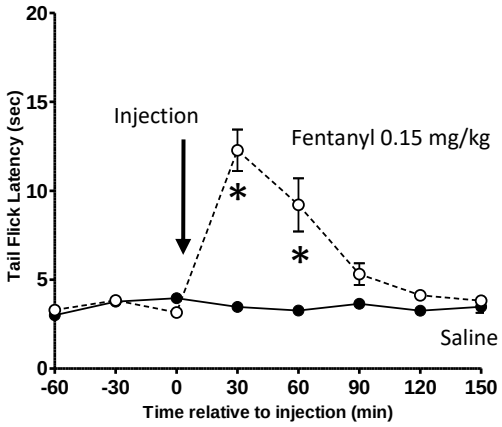


Figure 3.19: Acute fentanyl produces significant, yet transient, antinociception to a thermal stimulus*. Acute fentanyl (0.15 mg/kg) produced an increase in tail flick latency (30 & 60 min). Saline however produced no increase or decrease in tail flick latency. Fentanyl antinociception was transient and tail flick latency in fentanyl treated mice returned to baseline by 120 minutes post fentanyl administration. * indicates significance from pre-injection baseline values where p<0.05. Statistical comparison made by a Repeated Measures One-way ANOVA. N=6 for all groups.*

3.5 Discussion

3.5.1 Relative Potency of Opioid Agonists

One aim of this chapter was to determine the relative potencies of four opioid ligands *in vivo* using the physiological outputs of respiratory depression and antinociception. Morphine is the prototypic opioid agonists due to its prevalence as an analgesic and because morphine forms one of the major metabolites of heroin. As such, morphine is often used as the standard agonist in opioid experiments; in this case a dose of morphine (10 mg/kg) was chosen as the primary challenge dose due to the significant amount of respiratory depression it induced. As further experiments would be attempted to induced tolerance against this dose of morphine, an appreciable power of effect was required to assess partial levels of tolerance that would not be detectable if the dose of morphine chosen did not produce significant respiratory depression. It is also known that this dose of morphine does not induce hyperlocomotion (Hill et al., 2016).

Therefore, the range of doses chosen for oxycodone, methadone and fentanyl were designed to find a dose that was approximately equipotent with morphine 10 mg/kg at depressing respiration. Estimations of dose ranges for investigation were taken from the existing literature as well as existing knowledge within the laboratory itself.

The rank order of opioid agonist potencies in respiratory depression derived from the data within this chapter is as follows: ***Fentanyl > oxycodone > methadone = morphine***. However, when equi-potent doses for respiratory depression were used to determine antinociceptive potency of each opioid the rank order changes to: ***Fentanyl > oxycodone > morphine > methadone***.

The fact that methadone and morphine required the same dose to produce equal levels of respiratory depression was unexpected, due to multiple publications finding methadone to have greater efficacy and potency at the MOPr and *in vivo* than morphine (Borgland et al., 2003, McPherson et al., 2010, Saidak et al., 2006, Selley et al., 1998). Methadone has a greater potency than morphine for agonist stimulation of G-protein activation, arrestin recruitment and phosphorylation of the MOPr c-terminus (specifically serine-375) (McPherson et al., 2010). Additionally, methadone has an approximately 2-fold lower K_D value for the MOPr (McPherson et al., 2010, Selley et al., 1998). However, these are derived *in vitro* and cannot explicitly predict *in vivo* potency. The scant evidence comparing in vivo potency has been performed in rats, exclusively in antinociception assays and has concluded that methadone is indeed more potent than morphine (Altarifi et al., 2015, Lemberg et al., 2006).

More perplexing is the result that methadone appears to produce less antinociception than morphine when administered at doses that are equipotent at producing respiratory depression. As previously mentioned (See Section 3.3.2) this may well have occurred due to the methadone antinociception experiments being performed at a different time than those for other agonists; such batch variations in the responses of mice may well have produced this apparently aberrant result.

Oxycodone was determined to be approximately 3-fold more potent than morphine at depression respiration and similarly 3-fold more potent than morphine at inducing antinociception. These results agree with other publications (Raehal and Bohn., 2011, Jacobs et al., 2017).

Similarly, fentanyl was determined to be approximately 67-fold more potent than morphine at depressing respiration, a difference in potency that is supported by the existing literature (Kuo et al., 2015, Pereira et al., 2001). Peak levels of antinociception were not significantly different between morphine and fentanyl, but the half-life of fentanyl was markedly shorter with fentanyl, producing a far more transient antinociceptive response in mice. The relatively short half-life of fentanyl is also well documented in the existing literature (Kuo et al., 2015, Pereira et al., 2001).

Overall, the relative potencies of these four agonists at depressing respiration generally reflect the published literature on the potencies of morphine, oxycodone, methadone and fentanyl. The relatively low potency of methadone to induce antinociception was surprising, as one would expect methadone to at least be equipotent at the same dose as morphine given the results regarding respiratory depression, or based on the existing literature, methadone should have been more potent. A thorough repeat of this observation is required before a firm conclusion can be drawn.

3.5.2 Opioid Receptor Agonism by Oxycodone

Morphine and methadone are both though to be relatively selective opioid agonists for the MOPr over the DOPr and KOPr. However, oxycodone has previously been suggested as a having agonist activity at both the DOPr (Yang et al., 2016) and KOPr (Ross and Smith, 1997, Nielsen et al., 2007), with the agonist activity at the DOPr potentially forming a significant component of oxycodone induced respiratory depression (Yang et al., 2016). However, specific antagonism of both the DOPr and KOPr failed to significantly inhibit oxycodone induced respiratory depression, whereas the non-selective opioid antagonist naloxone fully inhibited oxycodone induced respiratory depression. These results were also seen with morphine and would indicate that the MOPr is the primary mediator of opioid induced respiratory depression with little to no contribution from DOPr or KOPr in the respiratory nuclei.

3.5.3 Fentanyl Resistance to Naloxone Antagonism

Administration of naloxone is the primary means of rescuing patients that are undergoing an opioid overdose event (CDC, 2015, CDC, 2016). Suggestion that fentanyl is relatively resistant to antagonism by naloxone (CDC, 2015, CDC, 2016) is a significant concern with the rise in opioid overdoses, both fatal and non-fatal, that involve fentanyl. At least a 10-fold higher dose of naloxone was required to reverse fentanyl respiratory depression compared to a dose of morphine that was equipotent at depressing respiration.

Classical pharmacological theory states that the same dose of an antagonist will equally antagonise equipotent doses of agonists acting at the same receptor in the same system. In this regard, fentanyl appears to function outside of classical pharmacology. Interestingly, whilst the *in vivo* and human literature frequently describes fentanyl as being 50-100 times more potent than morphine (Kuo et al., 2015, Pereira et al., 2001), *in vitro* data finds fentanyl to either be the same potency of morphine or only marginally more potent (<10 fold) (Pasternak and Snyder, 1975). Again, fentanyl appears to function as an opioid agonist in a way that is not classically definable with regards to potency.

Potentially, the difference in potency of fentanyl observed *in vitro* vs *in vivo* may be causally linked to the relative resistance of fentanyl to antagonism by naloxone. This may be due to unique pharmacokinetics in vivo that are not seen in *in vitro*, possibly relating the high lipophilicity of fentanyl, allowing fast on-off rates and competitive re-binding at the MOPr that prevents naloxone binding. A great deal of further work is needed to elucidate how fentanyl achieves potency *in vivo* and is resistant to naloxone.

4.0 Development of Tolerance to Opioids

4.1 Introduction

Tolerance is defined in the Diagnostic and Statistical Manual of Mental Disorders (DSM-V), as, on repeated drug taking, the requirement for markedly increasing the dose of a drug to achieve the same level of desired effect OR a marked decrease in the effect of the drug when the original dose is administered (Battle, 2013). Tolerance is an important pharmacological mechanism regarding opioid use. Tolerance is observed in opioid users, with large increases in the dose of opioid required to induce euphoria after repeated use (Hickman et al., 2008a, Warner-Smith et al., 2001).

Additionally, there is evidence to suggest that tolerance to opioids is important in their clinical use as analgesics, with reports of dose escalation or opioid rotation required due to the development of tolerance (Chapman and Hill, 1989, Cherny et al., 1995, Inturrisi et al., 1990). However, this is not universally accepted, with some clinicians stating that tolerance to the analgesic effect of opioids is not observed clinically and as such is not a problem (Arner et al., 1988, Brescia et al., 1992, Rowbotham et al., 2003, Schug et al., 1992). These views are often obfuscated by patient recovery or decline, poor record keeping of prescriptions as well as opioid rotation being initiated early enough to prevent defined tolerance (Collett, 1998, Foley, 2003, McQuay, 1999, Mercadante, 1999). Antinociceptive tolerance however, is readily observed through *in vivo* experimentation in rodents and higher order animals such as primates (Bohn et al., 2002, Dighe et al., 2009, Lin et al., 2012, Mohammed et al., 2013, Paronis and Bergman, 2011).

Opioid tolerance is often investigated with regard to opioid-induced antinociception, given that opioids are used primarily as analgesics clinically. The development of tolerance to opioid antinociception in animal models is, broadly speaking, an accepted phenomenon. The same cannot be said regarding tolerance to other opioidergic effects. With regards to opioid induced respiratory depression there are conflicting reports that tolerance does (Mohammed et al., 2013, Roerig et al., 1987) or does not (Kishioka et al., 2000, Ling et al., 1989) occur.

In addition to varying reports on the development of opioid tolerance, there is considerable variance within the methodology used to both induce and assess tolerance. It is not uncommon for the same opioid used to induce tolerance also being used acutely to assess the degree of tolerance (Jacob et al., 2017, Hull et al., 2013).

However, different opioid agonists will occupy different levels of the available receptor population following administration (Emmerson et al., 1994). In order to compare the overall degree of tolerance following prolonged administration of an opioid, a standard reference is required. This thesis utilised an acute morphine challenge (10 mg/kg) as the standard opioid challenge. Administering the same dose of morphine should occupy the same proportion of opioid receptors and so changes in acute responses to morphine can be directly compared following prolonged treatment with different opioid agonists.

4.1.1 Chapter Aims

The aims of this chapter were:

(i) To characterise multiple methods to induce tolerance to morphine

(ii) To develop drug treatment protocols to induce tolerance with prolonged or repeated administration of oxycodone, methadone and fentanyl

4.2 Morphine Induced Tolerance to Morphine

4.2.1 Acute Tolerance to Morphine

Two groups of CD-1 mice were repeatedly administered a dose of morphine (10 mg/kg i.p.), with injection 3 h apart, up to a total of three doses. One group of mice had respiration measured after each dose of morphine and the other group had tail flick latency measured after each dose of morphine. Mice that had respiration recorded whilst breathing 95% air/5% CO_2 showed a significant decrease in MV following all three doses of morphine (Fig. 4.1A-B). However, the final dose of morphine showed a significantly reduced depression of MV compared to the first dose (Fig. 4.1C). Respiration had returned to baseline values for all mice prior to each dose of morphine following the first.

Mice that had tail flick latency measured showed significant antinociception over the entire observation period (15-45 min post morphine) following the first dose of morphine. The increase in tail flick latency was significantly reduced following both the second and third doses of morphine (Fig. 4.1D). The third and final dose of morphine also showed a significantly reduced tail flick latency compared to the second morphine dose after 45 min (Fig. 4.1D). Tail flick latency had returned to baseline values for all mice prior to each dose of morphine following the first and there was no evidence of hyperalgesia induced by repeated injections.

4.2.2 Development of Tolerance with Twice Daily Doses of Morphine

A single group of CD-1 mice received twice daily injections of morphine (10 mg/kg i.p.) for 5 days. Baseline respiration was measured each day in the morning and the effect of morphine on respiration was measured each afternoon following the second injection of morphine (except for day 1 when it was measured after the first injection). See Methods section 2.4.1.2. Baseline MV for all mice was consistent across the 5 days of experimentation and showed no change. Similarly, the decrease in MV seen following morphine injection remained the same throughout the experiment (Fig. 4.2A).

A second group of CD-1 mice received the same twice daily injections of morphine as outlined above. However, instead of measuring respiration, mouse tail flick latency was measured. Baseline tail flick latency remained consistent across all days of the experiment. Morphine administration on days 1-2 caused a significant increase in tail flick latency, however morphine injections on days 3-5 did not produce a significant increase in tail flick latency over baseline (Fig. 4.2B). Baseline tail flick latency remained constant throughout the experiment, with no evidence of hyperalgesia.

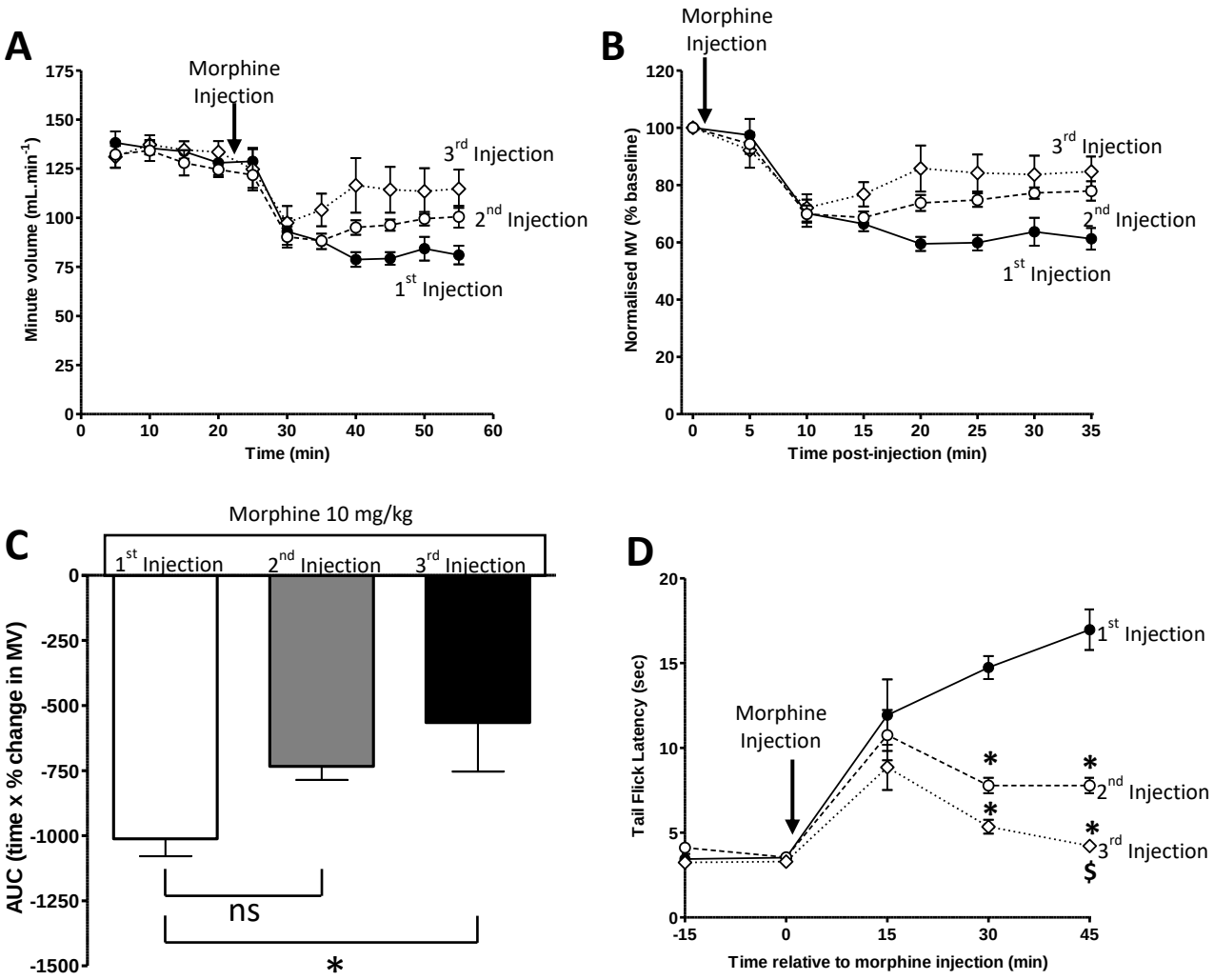


Figure 4.1: Development of acute tolerance to morphine by repeated frequent injections*. Morphine 10 mg/kg was administered at 3 h intervals and changes in MV (**A**, **B**, **C**) and tail flick latency (**D**) measured. Both 2nd and 3rd morphine injections showed smaller decreases in MV (**A**, **B**) however overall only the decrease following the 3rd injection were statistically significant (**C**). The tail flick latency following both the 2nd and 3rd injections were statistically lower compared to the 1st injection (**D**). Tail flick latency following the 3rd injection was also statistically lower than tail flick latency following the 2nd injection at 45 min post morphine. * indicates significance from 1st injection and $ indicates significance from 2nd injection with p<0.05. Statistical comparison made using a One-Way ANOVA with Bonferroni's comparison (**C**) or a Repeated Measures Two-way ANOVA (**D**). ns indicates non-significance. N=6 for all groups.*

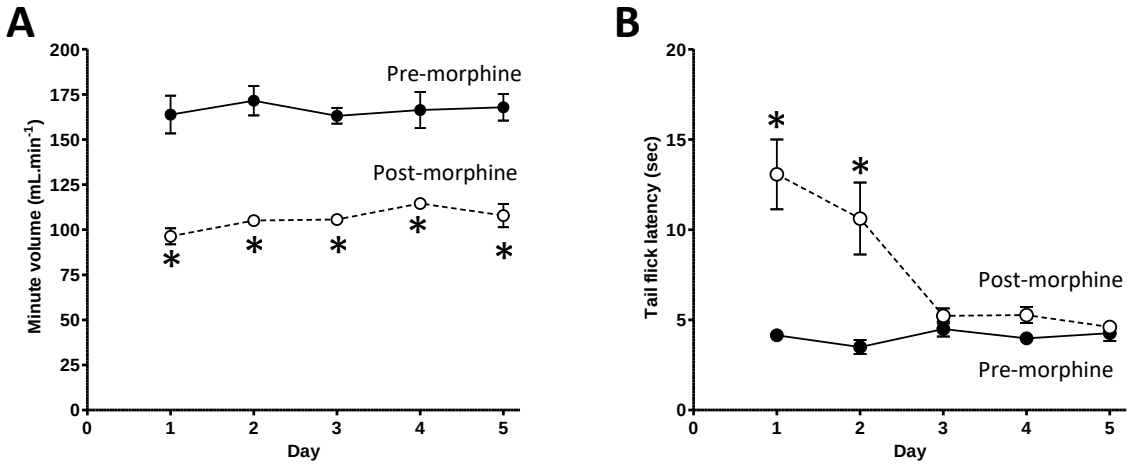


Figure 4.2: The effect of twice daily injections of morphine on respiratory depression and tail flick latency. *(**A**) With twice daily morphine 10 mg/kg, administered 12 h apart there was no change in morphine depression of MV over 5 days. (**B**) With the same twice daily morphine treatment there was a significant decrease in morphine induced tail flick latency enhancement on days 3-5. * indicates significance from pre-morphine baseline values p<0.05. Statistical comparison made using a Two-way ANOVA with Bonferroni's comparison. N=6 for all groups.*

4.2.3 Development of Tolerance with Continuous Exposure to Morphine Through an Osmotic Mini-Pump

Intermittent morphine administration over a prolonged period is sufficient to induced tolerance to morphine-induced antinociception but not to morphine-induced respiratory depression. A previous publication has discussed the difference between intermittent and continuous morphine exposure (Dighe et al., 2009) and concluded that continuous morphine produces greater tolerance.

Osmotic mini-pumps were implanted in mice for 6 days and provided a continuous infusion of morphine (45 mg/kg/day s.c.). Prior to implantation of osmotic mini-pumps mice also received priming injections of morphine (See Materials and Methods section 2.4.1). Control mice were implanted with pumps filled with saline and received saline priming injections.

Neither saline pump nor morphine pump implanted mice showed any significant changes in MV over the course of the 6 d treatment (Fig. 4.3A). However, on day 6, mice implanted with a saline pump showed a significant decrease in MV following an acute challenge of morphine (10 mg/kg i.p.) (Fig. 4.3B-C). Mice implanted with a morphine pump for 6 d did not show a decrease in MV following an acute challenge of morphine or an acute challenge of saline (Fig. 4.3B-C). Therefore, 6 d continuous treatment with morphine significantly attenuated the ability of acute morphine to depress MV. The response of morphine pump-implanted mice to saline or to morphine were not statistically different from each other (Fig. 4.3D).

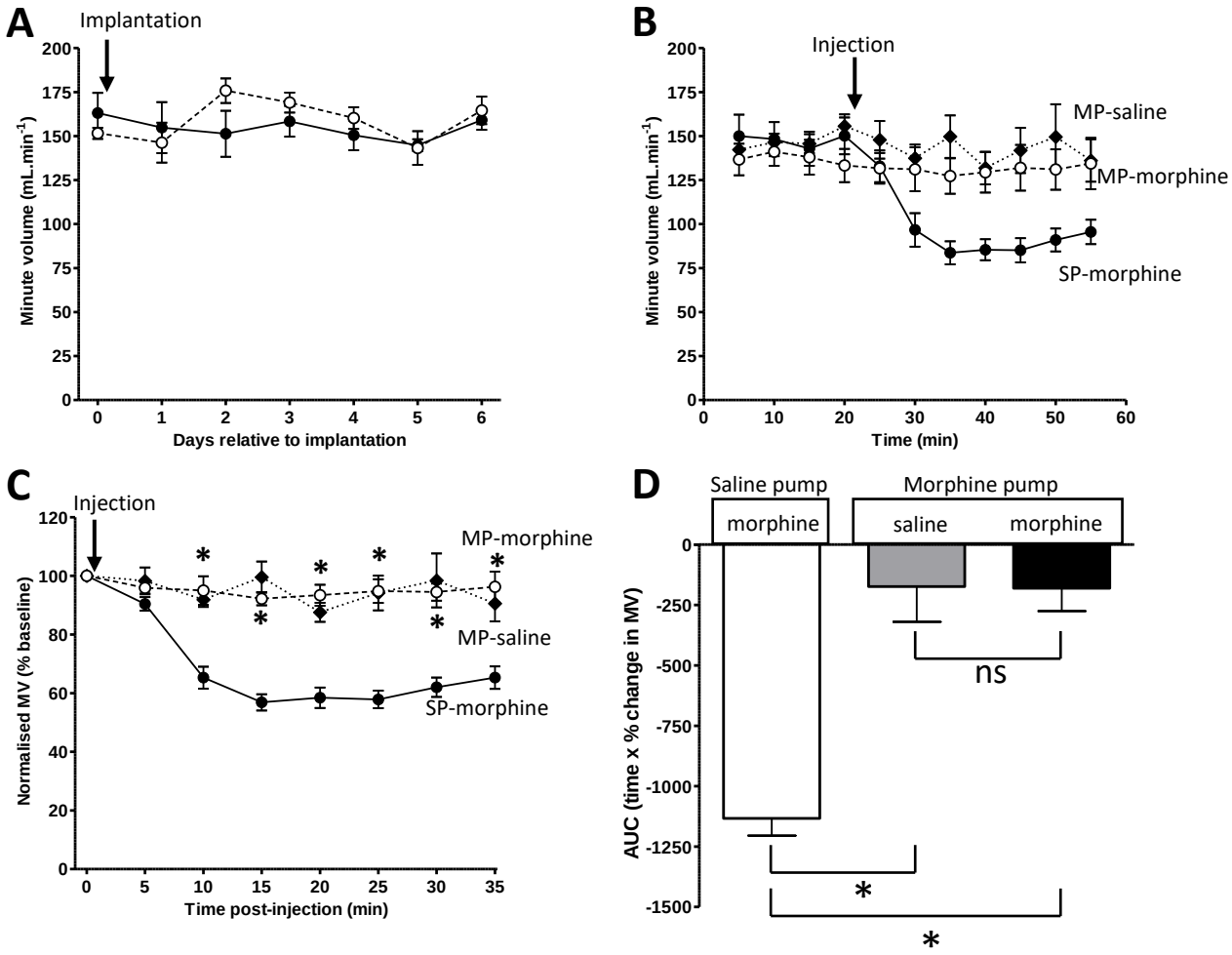


Figure 4.3: Prolonged continuous administration of morphine decreased acute morphine-induced respiratory depression*. Mice received 3 x priming injections of morphine (100 mg/kg i.p.) followed by 6d morphine pump (MP) treatment (45 mg/kg/day s.c.) or saline pump (SP) control treatment. (**A**) neither morphine nor saline pump implantation caused significant changes in baseline MV over the 6d treatment. (**B** and **C**) Acute morphine (10 mg/kg i.p.) caused a significant and prolonged depression of MV in saline pump implanted mice. Neither saline nor morphine caused a changed in MV in morphine pump implanted mice. (**D**) Area under the curve (AUC) analysis of (**C**) demonstrates significant reduction in morphine depression of MV in morphine pump implanted mice. * indicates significance from saline pump implanted mice receiving morphine with p<0.05. ns indicates non-significance. Statistical comparison made using a Two-way ANOVA (**C**) One-way ANOVA with Bonferroni's comparison **(D).** N=7 for all groups.*

4.2.4 Tolerance to Morphine Induced by Subcutaneous Morphine Pellet Implantation

In addition to providing prolonged continuous morphine through osmotic mini-pumps, mice were also implanted subcutaneously on the dorsal flank with a 75 mg morphine pellet for a total of 6 d (See Materials and Methods section 2.4.1). In contrast to mice implanted with morphine filled osmotic mini-pumps, mice implanted with morphine pellets showed a significant decrease in MV on days 1-3 post morphine pellet implantation (Fig. 4.4A). However, MV returned to pre-implantation baseline levels on days 4, 5 & 6. Placebo pellet-implanted mice showed no change in baseline MV over the 6 d (Fig. 4.4A). When placebo pellet implanted mice received an acute challenge dose of morphine (10 mg/kg i.p.) on day 6 this significantly depressed MV (Fig. 4.4B-C). In contrast, an acute challenge dose of morphine administered to morphine pellet-implanted mice did not decrease MV throughout the period of observation (Fig. 4.4B-C). The decrease in MV following acute challenge morphine was significantly attenuated in morphine pellet implanted mice when calculated as AUC (Fig. 4.4D).

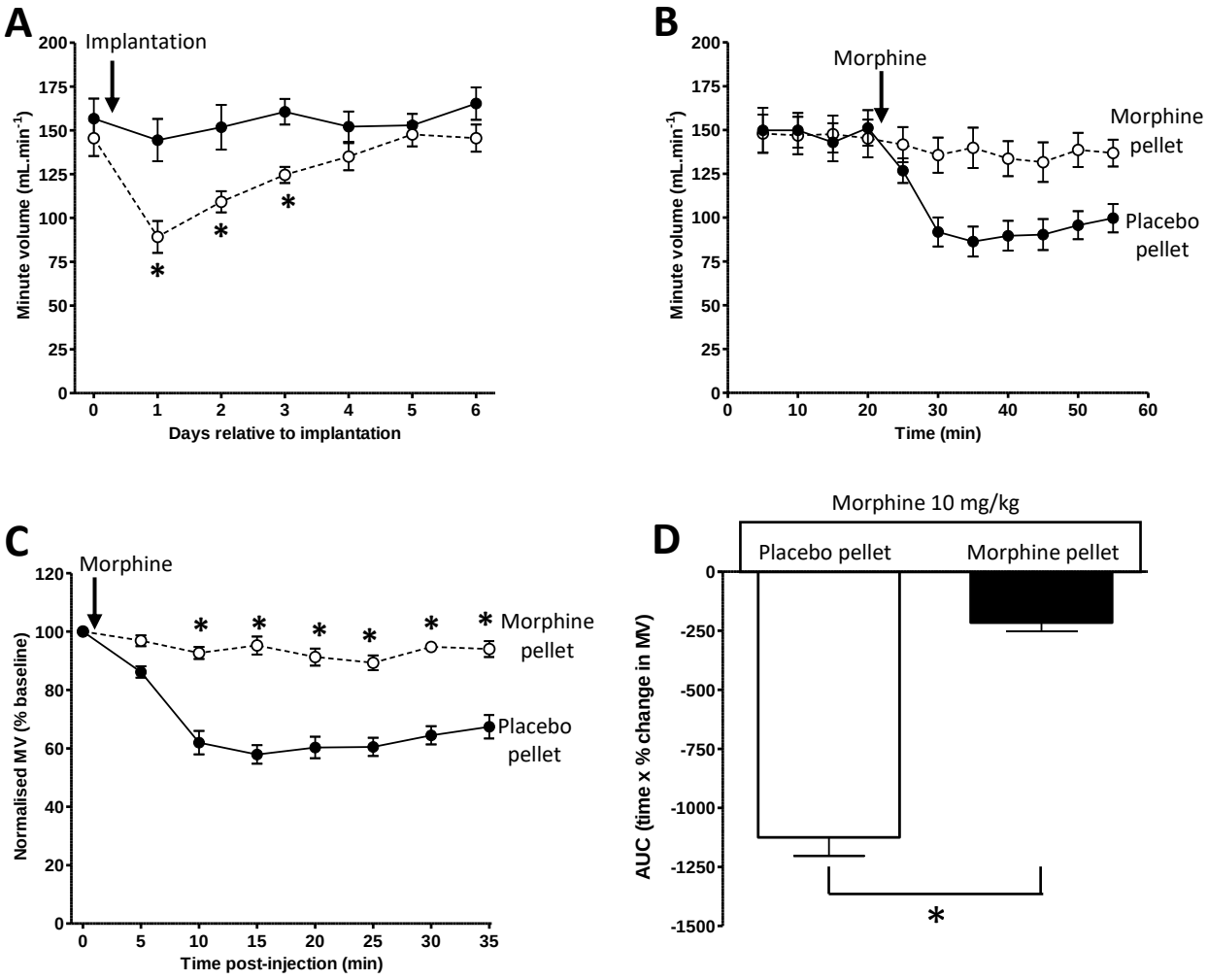


Figure 4.4: Prolonged continuous administration of a morphine pellet decreased acute morphine-induced respiratory depression*. Mice received a 6d morphine pellet treatment (75 mg s.c.) or placebo pellet control treatment. (**A**) placebo pellet implantation did not cause any significant changes in baseline MV over the 6d treatment. Morphine pellet implantation caused significant depression of MV on days 1-3 compared to pre-implantation baseline. (**B** and **C**) Acute morphine (10 mg/kg i.p.) caused a significant and prolonged depression of MV in placebo pellet-implanted mice. Morphine did not cause a significant decrease of MV in morphine pellet-implanted mice. (**D**) Area under the curve (AUC) analysis of data in (**C**) demonstrates the significant reduction of morphine MV depression in morphine pellet-implanted mice. (**A**) * indicates significance from pre-implantation baseline. (**C & D**) * indicates significance p<0.05 from placebo pellet implanted controls. Statistical comparison made using a repeated measures Two-way ANOVA (**A**), a Two-way ANOVA (**C**) or an unpaired two-tailed Student's T-test (**D**). N=6 for all groups.*

4.3 Development of Tolerance to Morphine with Continuous Exposure to Oxycodone

Oxycodone is not available in the form of a pellet and there is a scarcity of research on multiple injection protocols for oxycodone that relate to oxycodone-induced respiratory depression. Given the data collected previously on the development of tolerance to morphine respiratory depression (Section 4.2), the use of an osmotic mini-pump implanted on the dorsal flank of CD-1 mice for the same period of 6d period was considered the best approach to induce tolerance.

Previously, data presented on acute doses of oxycodone have shown it to be approximately 3 times more potent than morphine at inducing respiratory depression (See Chapter 3 Section 3.2). However, there is also evidence to suggest that mouse metabolism of oxycodone is faster than that of morphine (Raehal and Bohn, 2011). To account for both variables, three prolonged oxycodone treatments were used. These will hereafter be referred to as 'low oxycodone treatment', 'medium oxycodone treatment' and 'high oxycodone treatment'. 'Saline treatment' will refer to saline pump implanted mice (See Materials and Methods section 2.4.1).

Morphine (10 mg/kg i.p.) administered to saline pump-implanted mice caused a rapid and significant decrease in MV 10 min following morphine administration that remained at this level for the rest of the observation period (Fig. 4.5B). This can be seen when these data are expressed either as raw MV (Fig. 4.5B) or as percent of baseline MV (Fig. 4.5C). The saline pump, morphine challenged control group is the same in (Figs. 4.5, 4.6 4.7 & 4.8) as this control was conducted simultaneously with all three oxycodone pump treatments.

Low oxycodone pump implantation did not result in a decrease in MV during the 6 d pump treatment period (Fig. 4.5A). Acute morphine (10 mg/kg i.p.) administered to mice that had received low oxycodone treatment for 6d decreased MV to the same degree at 5 and 10 min post morphine administration. However, the decrease in MV at 15-35 min post morphine administration was reduced in low oxycodone pump implanted mice (Fig. 4.5B). This was true for both raw MV (Fig. 4.5B) and percent of baseline MV (Fig. 4.5C). Additionally, the overall decrease in MV following morphine administration, calculated as AUC, was significantly reduced by low oxycodone treatment. For combined data, see Fig. 4.8.

Medium oxycodone pump treatment resulted in a significant decrease in mouse MV on day 1 post pump implantation, before returning to baseline MV levels on subsequent days (Fig. 4.6A). Morphine (10 mg/kg i.p.) administered to mice that had received medium oxycodone treatment showed no decrease in MV or percent of baseline MV at all time points following morphine administration (Fig. 4.6B-C) compared to saline pump control. The overall response to morphine was significantly reduced in medium oxycodone treatment mice when calculated as AUC. For combined data, see Fig. 4.8.

High oxycodone pump treatment resulted in a significant decrease in mouse MV on days 1 ad 2 post pump implantation, before returning to baseline MV levels on subsequent days (Fig. 4.7A). Morphine (10 mg/kg i.p.) administered to mice that had received high oxycodone treatment showed no decrease in MV or percent of baseline MV at all time points following morphine administration (Fig. 4.7B-C) compared to saline pump control. High oxycodone pump implanted mice were also challenged with saline to demonstrate that a severe and prolonged oxycodone treatment did not have additional effects on normal respiratory parameters in response to vehicle. Saline did not change MV in high oxycodone treatment mice (Fig. 4.7B-C). The overall response to morphine was significantly reduced in high oxycodone treatment mice when calculated as AUC (Fig. 4.8). The response to morphine in medium or high oxycodone pump treatment mice was not significant when compared to the saline response in high oxycodone treatment mice (Fig. 4.8).

These data suggest that whilst a low oxycodone treatment significantly reduces the ability of morphine to depress respiration, a dosing regimen of either the medium or high oxycodone treatment are required to induce severe tolerance to morphine respiratory depression.

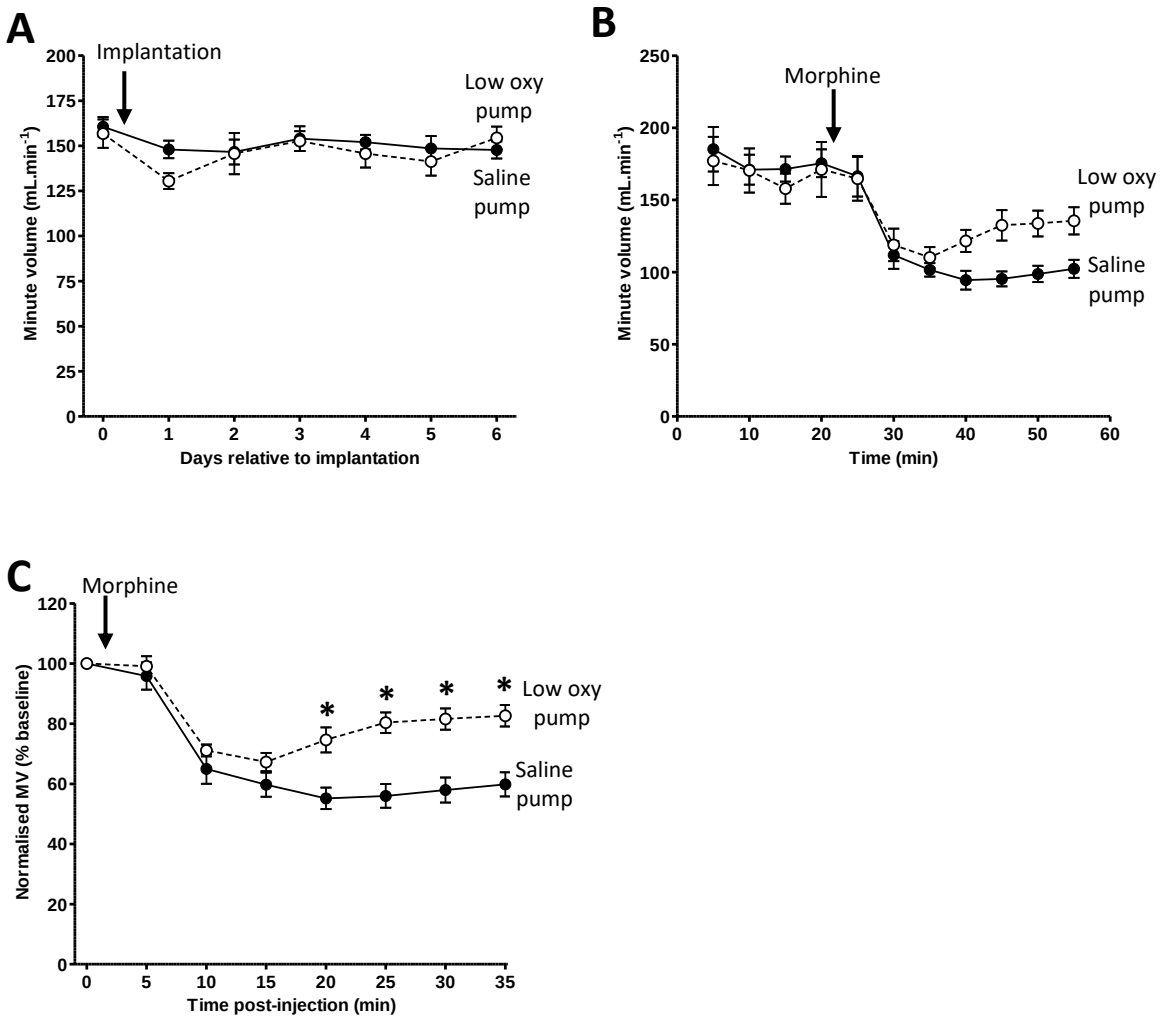


Figure 4.5: 6d Low oxycodone treatment decreased acute morphine-induced respiratory depression*. Mice received 3 x priming injections of oxycodone (30 mg/kg i.p.) followed by 6 d low oxycodone pump treatment (25 mg/kg/day s.c.) or saline pump control treatment. (**A**) neither low oxy nor saline pump implantation caused significant changes in baseline MV over the 6d treatment. (**B** and **C**) Acute morphine (10 mg/kg i.p.) caused a significant and prolonged depression of MV in saline pump implanted mice. Morphine produced a significant decrease of MV in low oxycodone pump implanted mice, though this was reduced compared to saline pump implanted mice. * indicates significance from saline pump implanted mice with p<0.05. Statistical comparison made using a repeated measures Two-way ANOVA with Bonferroni's comparison. N=7 for all groups.*

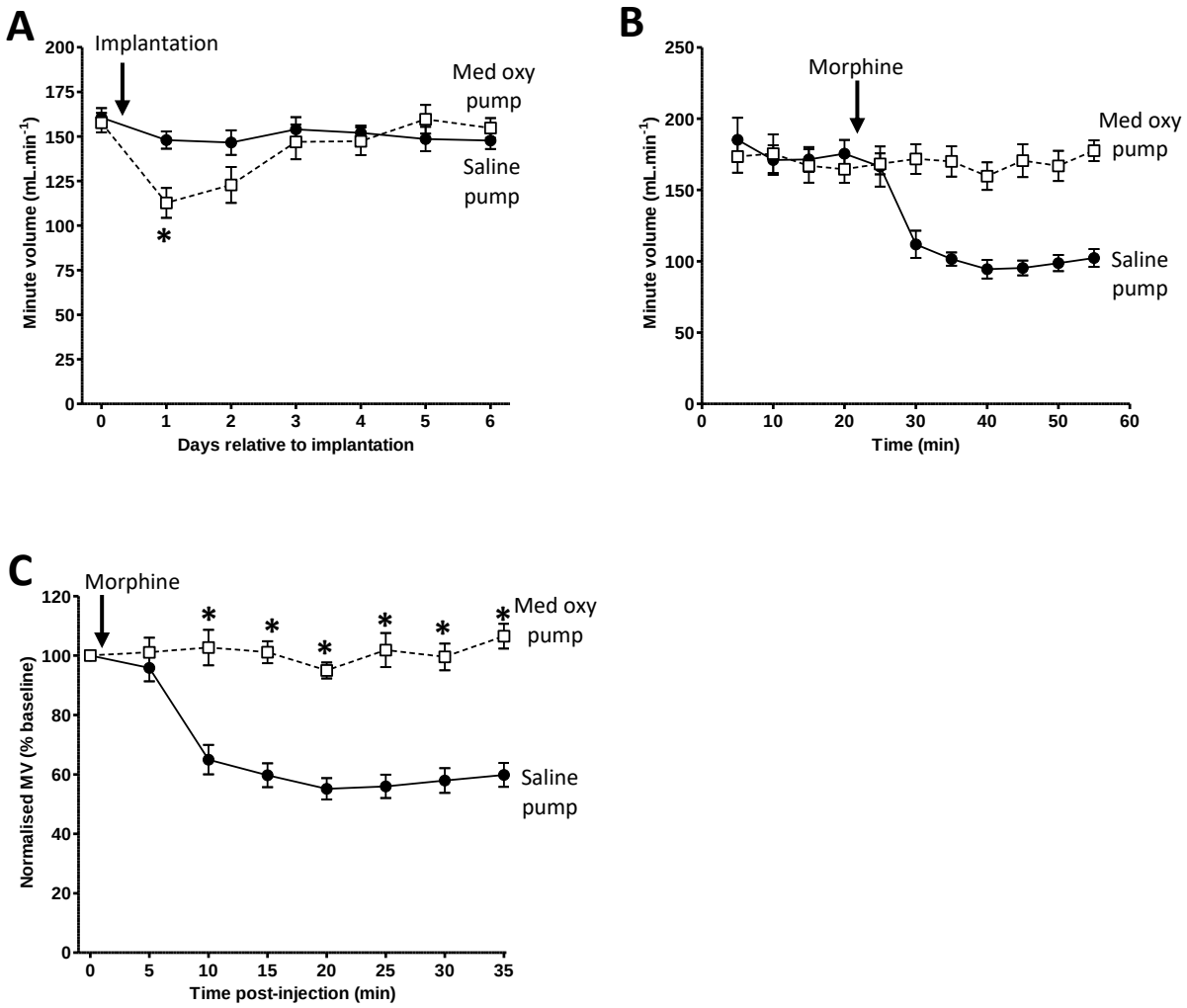


Figure 4.6: 6d Medium oxycodone treatment decreased acute morphine-induced respiratory depression*. Mice received 3 x priming injections of oxycodone (100 mg/kg i.p.) followed by 6 d medium oxycodone pump treatment (45 mg/kg/day s.c.) or saline pump control treatment. (**A**) saline pump implantation caused no changes in baseline MV. Med oxy pump implantation caused a significant decrease in MV on day 1 post pump implantation. (**B** and **C**) Acute morphine (10 mg/kg i.p.) caused a significant and prolonged depression of MV in saline pump implanted mice. Morphine caused no decrease of MV in medium oxycodone pump implanted mice. * indicates significance from saline pump implanted mice with p<0.05. Statistical comparison in **A**) is a repeated measures Two-way ANOVA with Bonferroni's comparison, in **B**) is a Two-way ANOVA with Bonferroni's comparison. N=7 for all groups.*

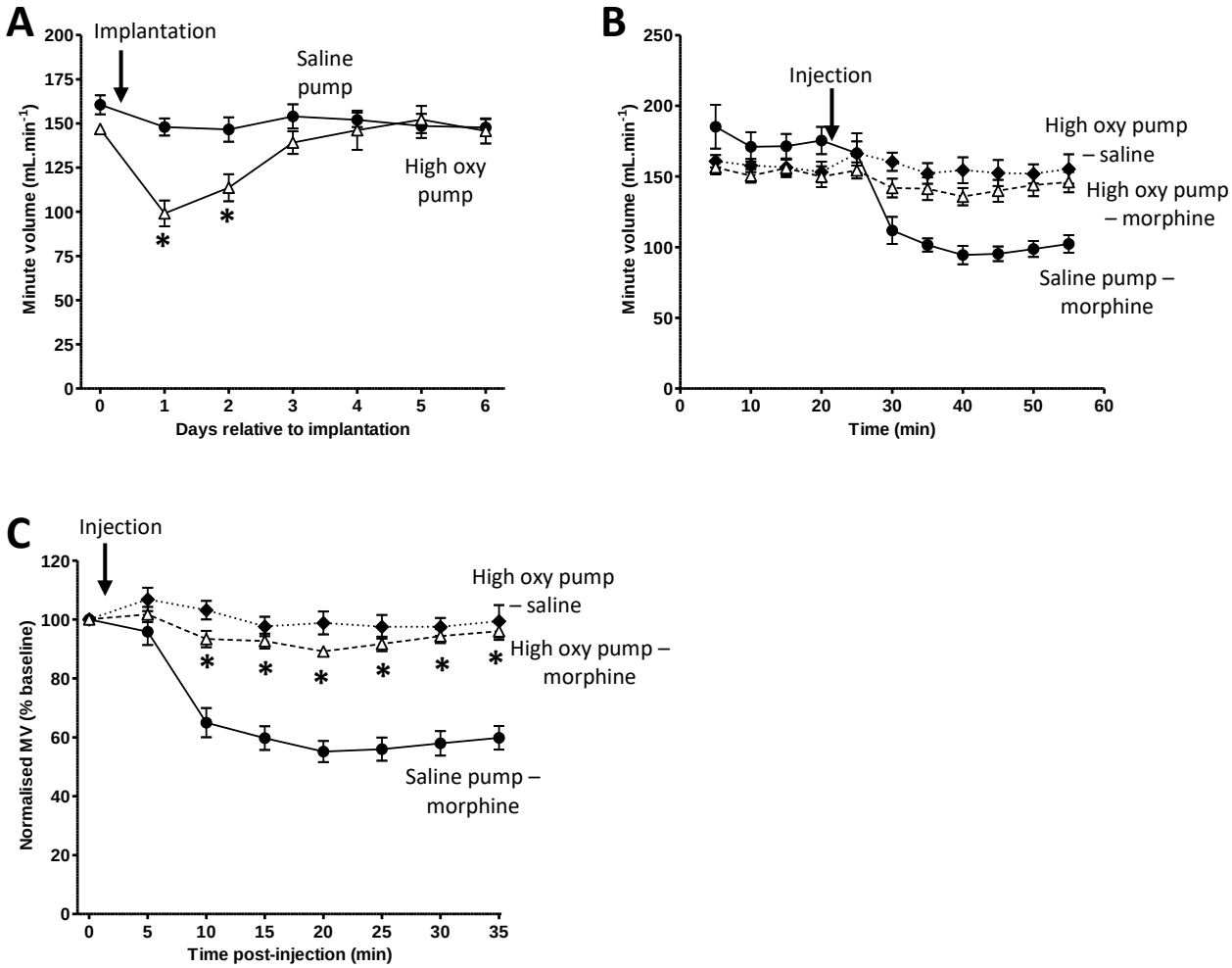


Figure 4.7: 6d High oxycodone treatment decreased acute morphine-induced respiratory depression*. Mice received 3 x priming injections of oxycodone (100 mg/kg i.p.) followed by 6 d high oxycodone pump treatment (120 mg/kg/day s.c.) or saline pump control treatment. (**A**) saline pump implantation caused no changes in baseline MV. High oxy pump implantation caused a significant decrease in MV on days 1-2 post pump implantation. (**B** and **C**) Acute morphine (10 mg/kg i.p.) caused a significant and prolonged depression of MV in saline pump implanted mice. Morphine caused no decrease of MV in high oxycodone pump implanted mice. Acute saline administered to high oxycodone pump implanted mice caused no change to mouse MV. * indicates significance from saline pump implanted mice receiving morphine with p<0.05. Statistical comparison made using a repeated measures Two-way ANOVA with Bonferroni's comparison (**A**), a Two-way ANVOVA with Bonferroni's comparison (**C**). N=7 for all groups.*

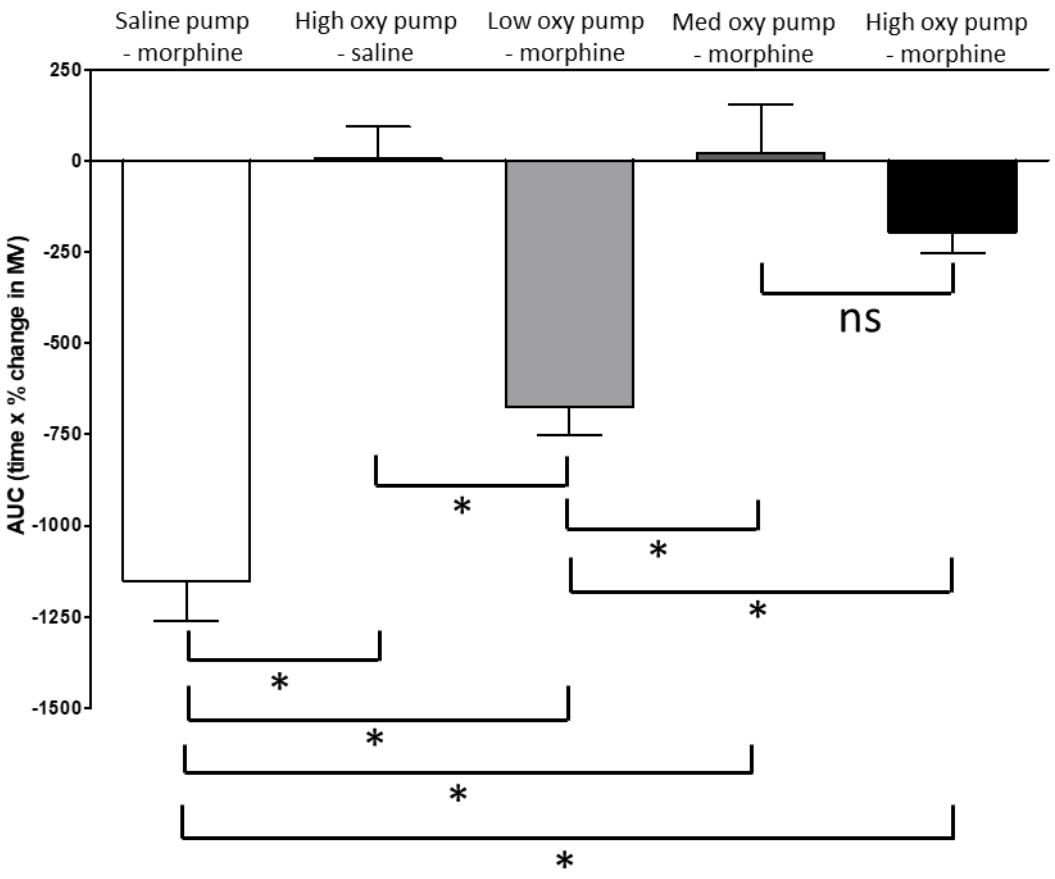


Figure 4.8: Prolonged oxycodone treatments reduce acute morphine-induced respiratory depression*. Area under the curve values calculated from Fig. 4.5C, 4.6C & 4.7C illustrate the overall change in MV following morphine (10 mg/kg i.p.) or saline administration. Saline pump implanted mice that received morphine showed a significant decrease in. Low oxycodone pump treatment significantly lowered morphine depression of MV, but significant depression of MV still occurred. Medium and high oxycodone treatment showed no significant decrease in MV over the observation period. Saline administered to high oxycodone pump implanted mice did not change MV. * indicates significance with p<0.05. ns = not-significant. Statistical comparison made using a One-way ANOVA with Bonferroni's comparison. Ns = not significant. N=7 for all groups.*

4.4 Development of Tolerance to Morphine with Continuous Exposure to Methadone

As with oxycodone, methadone is not available in the form of a pellet that can be implanted subcutaneously. Therefore, osmotic mini-pumps were used to administer methadone over a prolonged period of 6 d. Previous publications have used methadone-filled osmotic mini-pumps to generate tolerance (Quillinan et al., 2011), and so the same pump protocol for methadone was used as in that study. Comparison of acute morphine depression of MV in methadone pump implanted mice was made with mice implanted with saline pumps and also administered morphine (See Materials and Methods section 2.4.1).

The administration of a morphine challenge (10 mg/kg i.p.) to saline pump-implanted mice caused a rapid and significant decrease in MV, expressed either as raw MV (Fig. 4.9A) or as percent of baseline MV (Fig. 4.9B). The decrease in MV plateaued 10 min following morphine administration and was maintained for the remainder of the observation period. The same morphine challenge in 6 d methadone pump-implanted mice did not significantly alter MV over the observation period. When calculated as AUC, the ability of morphine to decrease mouse MV was almost completely attenuated in mice that had received methadone pump treatment for 6 d (Fig. 4.9C).

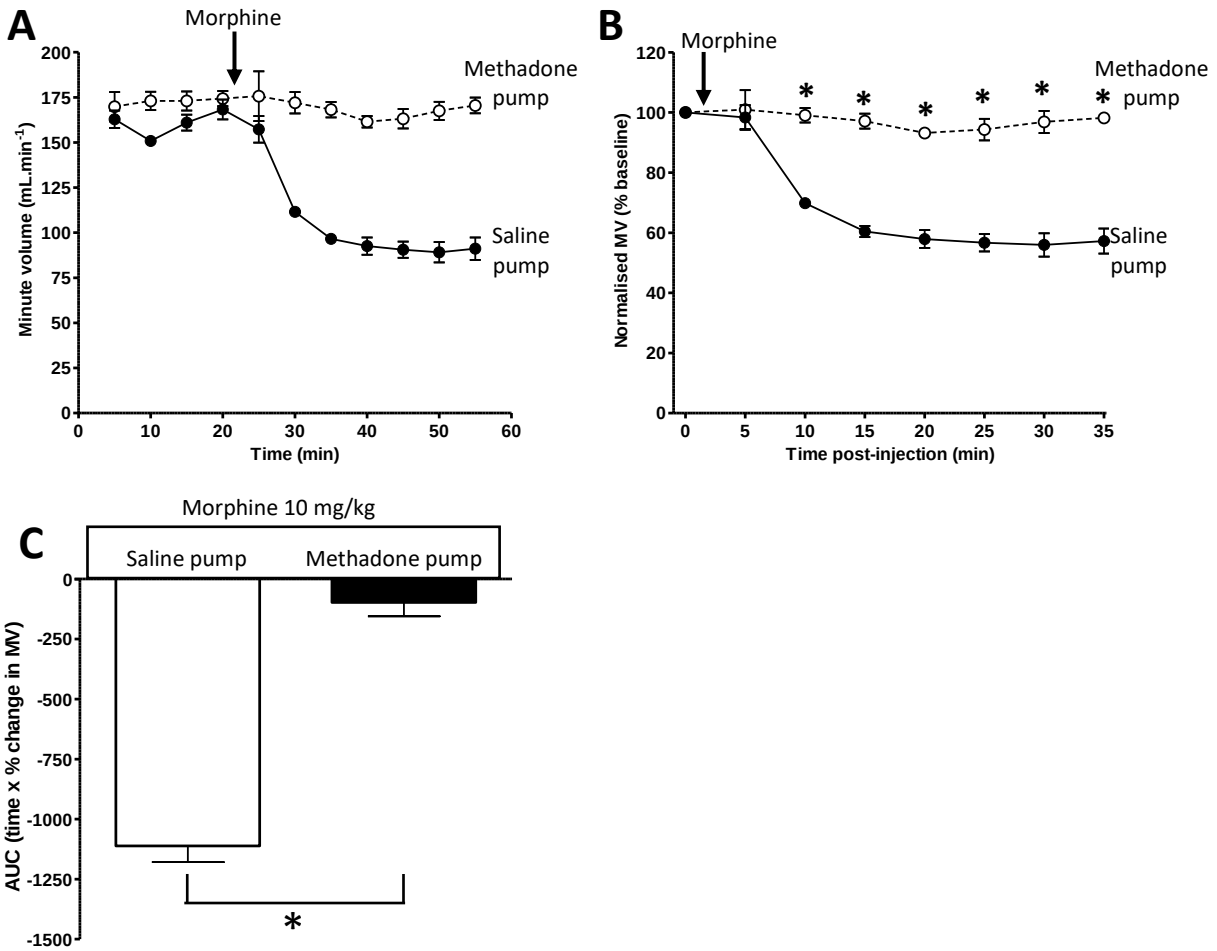


Figure 4.9: 6d Methadone pump treatment decreased acute morphine-induced respiratory depression*. Mice received 3 x priming injections of methadone (1 x 5 mg/kg and 2 x 7.5 mg/kg i.p.) followed by 6 d methadone pump treatment (60 mg/kg/day s.c.) or saline pump control treatment. (**A** and **B**) Acute morphine (10 mg/kg i.p.) caused a significant and prolonged depression of MV in saline pump implanted mice. Morphine caused no changed in the MV of methadone pump implanted mice. (**C**) Area under the curve (AUC) analysis of (**B**) demonstrates the significantly reduced depression of MV by morphine in methadone pump implanted mice. * indicates significance from saline pump implanted mice receiving morphine with p<0.05. Statistical comparison made using a Two-way ANVOVA with Bonferroni's comparison (**A**) an unpaired two-tailed Student's T-test (**C**). N=7 for all groups.*

4.5 Acute Tolerance to Fentanyl

Fentanyl is a short acting opioid agonist, and so a continuous administration of fentanyl in mice would be difficult to relate to the use of fentanyl in human addicts; who would not have significant levels of fentanyl in their system for prolonged periods, compared to morphine, oxycodone and methadone. To examine fentanyl tolerance, an acute tolerance paradigm was adapted from previously published work (Melief et al., 2010). In these experiments, the tail flick assay was used, with the increase in tail flick latency induced by fentanyl injection measured following two doses of fentanyl (0.15 mg/kg) administered 3 h apart, allowing for tail flick latency to return to baseline levels before the second dose on fentanyl was administered. (See Materials and Methods section 2.4.2).

The first dose of fentanyl caused a significant increase in mouse tail flick latency that peaked 30 min following fentanyl administration (Fig. 4.10A). The increase in tail flick latency caused by fentanyl administration then diminished until tail flick latency returned to baseline levels 120 min post the administration of the first fentanyl dose. A control group of mice that received saline instead of fentanyl did not show any change in tail flick latency.

In both groups of mice, fentanyl was administered 180 min after the initial injection of either fentanyl or saline. In the control group, fentanyl caused a significant increase in mouse tail flick latency that was not significantly different from the increase in tail flick latency seen in the group that had previously received fentanyl at time 0 min (Fig. 10a). Mice that received a second injection of fentanyl showed a significant increase in tail flick latency 30 min following the administration of the second fentanyl dose (time 210 min). The increase in tail flick latency then diminished back to baseline tail flick values by time 300 min. However, the overall effect of the second fentanyl dose was greatly reduced compared to that seen following the first fentanyl injection. AUC analysis of each peak shows this significant reduction (Fig. 4.10B).

The repeated acute administration of fentanyl therefore produced rapid tolerance to the fentanyl-induced antinociception in mice.

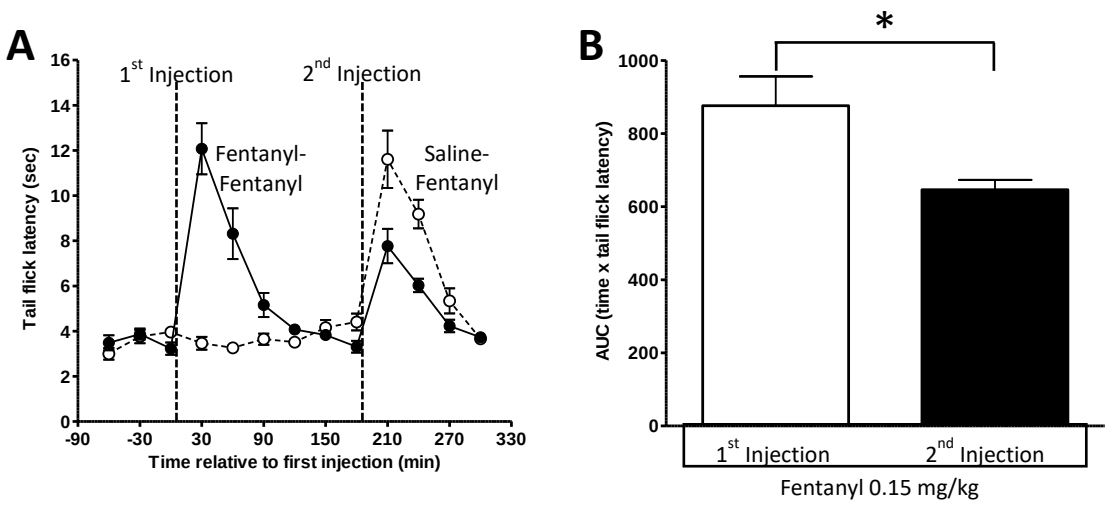


Figure 4.10: Acute repeated administration of fentanyl reduces fentanyl induced increase in tail flick latency*. Fentanyl (0.15 mg/kg i.p.) was administered at both the 1st and 2nd injection time points (solid circles, solid line) or saline was administered at the 1st injection and fentanyl at the 2nd (open circles, dashed line) with 3 h between injections. (**A**) Fentanyl produced a significant increase in tail flick latency following the first injection saline produced no change in tail flick latency. Mice injected with saline saw a significant increase in tail flick latency when administered fentanyl for the second injection, mice injected with fentanyl for the first and second injection saw a significant increase in tail f lick latency after the second injection though diminished from the first injection response (**A** and **B**) Mice that received two fentanyl injections showed a significant decrease in enhanced tail flick latency following the 2nd fentanyl injection. * indicates significance with p<0.05. Statistical comparison made using a paired two-tailed Student's Test. N=6 for all groups.*

4.6 Discussion

4.6.1 Morphine Tolerance

Following 6 d treatment with morphine filled osmotic mini-pumps, mice demonstrated a reduced response to a challenge dose of morphine, indicating that significant tolerance to morphine-induced respiratory depression had developed. Several previous publications have argued that tolerance does not develop to morphine respiratory depression. However, the time course of morphine administration was relatively short in those experiments, ranging from 3-8 hrs (Kishioka et al., 2000, Ling et al., 1989). Indeed, publications that utilised longer exposures to morphine have shown tolerance to morphine respiratory depression (Mohammed et al., 2013, Roerig et al., 1987), though not to the extent observed in this thesis. Again, the period of opioid administration was relatively short with Roerig et al (1987) utilising the longest opioid treatment of 72 h.

Twice daily repeated injections of morphine were sufficient to develop complete tolerance to morphine antinociception and yet did not significantly reduce the level of respiratory depression observed. These data would suggest that tolerance to different morphine mediated effects develops at different rates. Differential development of tolerance to discreet opioidergic actions has previously been postulated regarding the development of tolerance to morphine respiratory depression and morphine induced euphoria (White and Irvine, 1999), as well as previous work showing short morphine treatment over 8 h (i.v.) produced antinociceptive tolerance but not tolerance to respiratory depression (Ling et al., 1989).

However, repeated injections of morphine at short intervals did yield a slight but significant decrease in the level of morphine respiratory depression. These data would suggest that previous publications have failed to find tolerance to morphine respiratory depression due to the relatively short time course of the morphine dosing or the relatively low dosage of morphine. Intermittent injections of morphine, as previously reported (Dighe et al., 2009), appears to produce less pronounced tolerance to morphine than continuous morphine exposure, an effect that is more apparent when studying morphine-induced respiratory depression compared to morphine antinociception.

The difference in rate of tolerance development to morphine respiratory depression and morphine antinociception may be explained by the relative receptor levels in respiratory nuclei compared to the spinal cord. Additionally, there may be fundamental differences in the intracellular expression of proteins that mediate MOPr desensitization, internalisation or degradation that explain this difference in the rate of tolerance development.

4.6.2 Oxycodone Tolerance

Mice implanted with oxycodone pumps for 6 d showed varying levels of tolerance to morphine respiratory depression depending upon the dosing regimen used. Oxycodone is more potent than morphine (See Chapter 3 Section 3.2), in agreement with a previous publication (Jacob et al., 2017). However, the appropriate reduction in oxycodone pump dosing to account for this increased potency resulted in a decreased level of tolerance to a morphine challenge when compared to morphine pump treated mice. This may be due to the reduced half-life of oxycodone in mice compared to morphine (Raehal and Bohn, 2011), with the higher potency of oxycodone being overcome by the increased rate of oxycodone metabolism in mice.

Increasing the dose of oxycodone was able to produce a degree of tolerance to acute morphine respiratory depression that was comparable to the degree of tolerance seen following prolonged morphine pump or morphine pellet treatment.

Oxycodone abuse has been extremely prevalent in the USA (Inciardi et al., 2007, Kenan et al., 2012, Sgarlato and deRoux, 2015, Hedegaard et al., 2017b) and there is evidence to suggest that oxycodone addicted patients who initially received a therapeutic prescription of oxycodone, will in some situations subsequently switch their opioid use to heroin (Inciardi et al., 2007, Kenan et al., 2012, Hedegaard et al., 2017b). This appears to arise due to the patient's inability to procure a repeat oxycodone prescription or due to financial difficulties in affording the prescribed brand of oxycodone (Okie, 2010, Inciardi et al., 2007). These data suggest that cross tolerance between oxycodone and morphine does occur and this may help prevent the occurrence of accidental overdoses following a switch from oxycodone to heroin use. However, this would most likely depend heavily on the relative dosing habit of the individual. It is possible that dosing errors compounded with a relative lack of tolerance to morphine (as the rapidly produced metabolite of heroin) may result in accidental fatal overdoses.

4.6.3 Methadone Tolerance

Mice implanted with methadone pumps for 6 d also developed significant tolerance to morphine-induced respiratory depression. The use of pre-injections prior to all osmotic mini-pump surgeries was based upon previous research conducted within the laboratory that had replicated previous work using methadone filled osmotic mini-pumps (Quillinan et al., 2011). These pre-injection doses of methadone (or other opioids) are believed to raise opioid levels within the brain 'priming' the development of tolerance which is then maintained by the continuous flow of drug from the osmotic mini-pump. Previously, morphine blood and brain levels have been measured following morphine pellet implantation. These show an initial surge of morphine blood and brain levels in the first day following implantation that then decreases to a plateau. The pre-injection doses of methadone, oxycodone and morphine are thought to mimic this surge which seems to be important in facilitating the development of tolerance.

Indeed, methadone maintenance treatment in recovering opioid addicts has been shown to require dose escalation following initial prescription in order to sufficiently block euphoria obtained from on top heroin use. Methadone dose escalation is important in providing a block of both on top opioid euphoria and on top opioid respiratory depression. Many addicts are stabilized at higher doses before dose tapering is used to decrease the maintenance dose of methadone required.

4.6.4 Fentanyl Tolerance

Repeated fentanyl injections over a short time frame showed clear development of acute tolerance to fentanyl antinociception. This was consistent with data previously published and upon which this paradigm was based (Melief et al., 2010).

Further experimentation in this area would most likely focus on how well prolonged administration of morphine, oxycodone and methadone induce tolerance to fentanyl induced respiratory depression. However, at the time of experimentation this was not the aim of this investigation, and it is only light of recent events that the need for further data on fentanyl abuse has become a pressing matter.

So, whilst it is valuable to note that fentanyl generates moderate tolerance to itself when administered in rapid succession, this does not accurately reflect the consumption habits or dangers of fentanyl use amongst the opioid addicted community. This experimental protocol, does however allow a means to investigate the mechanisms that are recruited in the development of tolerance to fentanyl, and so is of great utility.

4.6.5 Overall Conclusion

These results demonstrate the ability to produce tolerance to opioid respiratory depression through short or long-term exposure protocols for all four opioid agonists. Differences in protocol have largely been driven by differences in both potency and metabolism of each opioid in the mouse. Despite this, the tolerance protocol for each opioid provides a sufficiently powerful decrease in opioid induced effect to measure alterations in sensitivity to each opioid when manipulated with other drugs of abuse or molecular inhibitors/potentiators.

5.0 Mechanisms of Opioid Tolerance

5.1 Introduction

The mechanism by which tolerance occurs at the level of the MOPr remains elusive. Not only is tolerance likely to be mediated by different signalling pathways (and therefore different kinases) for different opioid agonists, but a single opioid may recruit multiple kinases which results in tolerance (Williams et al., 2012). Additionally, there has been some discourse that suggests a difference in the mechanisms that occur during induction of opioid tolerance compared to the maintenance of prolonged opioid tolerance (Zhang et al., 2015, Christensen et al., 2000). Altogether this suggests that not only will there be opioid specific mechanisms of tolerance, but also temporally-specific mechanisms of tolerance, and potentially, there may also be multiple mechanisms of tolerance, specific to the modality of opioidergic effect, such as tolerance to opioid respiratory depression versus opioid analgesia.

There is a wealth of potential targets that may mediate opioid induced tolerance, this chapter focusses on three candidates that have previously been suggested as likely mediators of tolerance to opioids.

5.1.1 Protein Kinase C

Protein kinase C (PKC) is a downstream signalling kinase recruited through G-protein (G_q) activation that activates additional second messenger systems as well as potentially directly interacting with the MOPr. PKC has previously been suggested as the key signalling pathway for morphine desensitization at the MOPr, primarily through electrophysiological investigation (Bailey et al., 2009a, Bailey et al., 2009b, Hull et al., 2010). In particular, the PKC isoform PKCα was identified as primary mediator for MOPr desensitization by morphine (Bailey et al., 2009b).

Previously published research demonstrated the development of cellular tolerance in locus coeruleus slices taken from rats administered a continuous 3 d morphine treatment (Bailey et al., 2009a); this was reversible by PKC inhibition. Additionally, *in vivo* tolerance to morphine antinociception induced in mice through repeated administration of morphine over 8 hr was also reversible by PKC inhibition (Hull et al., 2010).

In addition to in vitro PKC manipulation, work has been conducted on mice that expressed constitutively active PKCα or PKCε prior to prolonged morphine administration (Lin et al., 2012). Mice transfected with both isoforms of constitutively active PKC developed tolerance to morphine antinociception significantly quicker than control DNA transfected mice. This further suggests that PKC activation is an important mediator of tolerance to morphine.

5.1.2 c-Jun N-terminal Kinase

Selective inhibition of c-JUN N-terminal Kinase (JNK) as well as JNK knock-out strains of mice has previously been used to investigate the role of JNK in the development of opioid tolerance. Selective knock-out of JNK2 has been shown to prevent the onset of tolerance to morphine, with JNK2 knock-out mice exhibiting little to no tolerance following repeated acute treatment with morphine (Melief et al., 2010). This result was also supported through experiments using the brain penetrant, broad spectrum JNK inhibitor SP600125 (Melief et al., 2010). SP600125 inhibits all JNK isoforms and was utilised to investigate the role of JNK in morphine tolerance.

5.1.3 G-protein Receptor Kinase

G-protein receptor kinases (GRKs) are a class of enzymes that regulate the activity of GPCRs through phosphorylation of key motifs within the C-terminus and intracellular loops of GPCRs. Phosphorylation by GRKs is considered necessary for the recruitment of arrestin to GPCRs which in turn limit or prevent the association of G-proteins and thus inhibit G-protein signalling (Vroon et al., 2006, Vayttaden et al., 2010, Williams et al., 2013). Opioid ligands have previously been characterised by their relative intrinsic efficacy to recruit G-protein versus arrestin (and by proxy GRK) signalling pathways (McPherson et al., 2010). These data form a core basis for hypothesising whether a given opioid ligand may be more, or less likely to produce tolerance through a G-protein or GRK/arrestin dependent mechanism.

McPherson et al (2010) described both morphine and oxycodone as having relatively poor intrinsic efficacy to recruit arrestin to the MOPr, compared to methadone and fentanyl which possess high intrinsic efficacy to recruit GRK/arrestin. Fentanyl in particular has been described as a strong activator of GRK in other published work (Morgan et al., 2014, Raehal et al., 2011, Terman et al., 2004, Macey et al., 2006), and even described as an arrestin biased opioid agonist (Schmid et al., 2017a).

5.1.4 Chapter Aims

The aims of this chapter were:

(i) To investigate the role of PKC activity in morphine, oxycodone, methadone and fentanyl induced tolerance

Calphostin C, a brain penetrant broad spectrum PKC inhibitor was used as a canonical inhibitor of PKC to investigate the role of PKC in the maintenance of opioid tolerance. However, tamoxifen was also utilised a PKC inhibitor. Tamoxifen is primarily known for its competitive oestrogen receptor antagonism, but it is also documented to inhibit PKC. The only known overlap in activity of calphostin C and tamoxifen is the inhibition of PKC. Utilising these two brain penetrant PKC inhibitors allowed a greater level of confidence that effects seen were due to PKC inhibition and not potential off target effects.

(ii) To investigate the role of GRK activity in fentanyl induced tolerance

With a relatively low amount of available compound 101, a GRK2/3 inhibitor, to investigate the role of GRK in opioid tolerance, fentanyl was identified as the most likely opioid agonist amongst the four investigated to produce tolerance through GRK/arrestin recruitment. Therefore, the available resource of compound 101 was directed towards use in fentanyl based experiments.

5.2 Morphine

5.2.1 Effect of Tamoxifen on Tolerance Induced by Prolonged Morphine Treatment

Male CD-1 mice received a prolonged treatment of either morphine or saline through implantation of an osmotic mini-pump for 6 d (See Materials and Methods section 2.4.1). Tamoxifen (0.6mg/kg) or vehicle (10% propylene glycol/90% saline) was administered 10 min prior to the recording of mouse respiration and a total of 30 min prior to the administration of an acute challenge dose of morphine (10 mg/kg) allowing time for inhibition of PKC as previously published (Hermenegildo et al., 1993). Tamoxifen has previously been shown by others within the laboratory to not depress respiration at this dose (See Appendix 2).

An acute morphine challenge administered to saline pump-implanted mice caused a decrease in MV in both vehicle or tamoxifen pre-treated groups (Fig. 5.1A & 5.1C). The decrease in MV was not significantly different between vehicle or tamoxifen pre-treated mice (Fig. 5.1E). The same acute morphine challenge administered to morphine pump-implanted mice, and mice pre-treated with vehicle, did not cause a decrease in MV (Fig. 5.1B & 5.1D).

However, a morphine challenge administered to morphine pump-implanted mice, pre-treated with tamoxifen, decreased MV significantly compared to vehicle control (Fig. 5.1B & 5.1D-E). This was not significantly different from the morphine-induced decrease in MV induced in saline pump implanted mice (Fig. 5.1E).

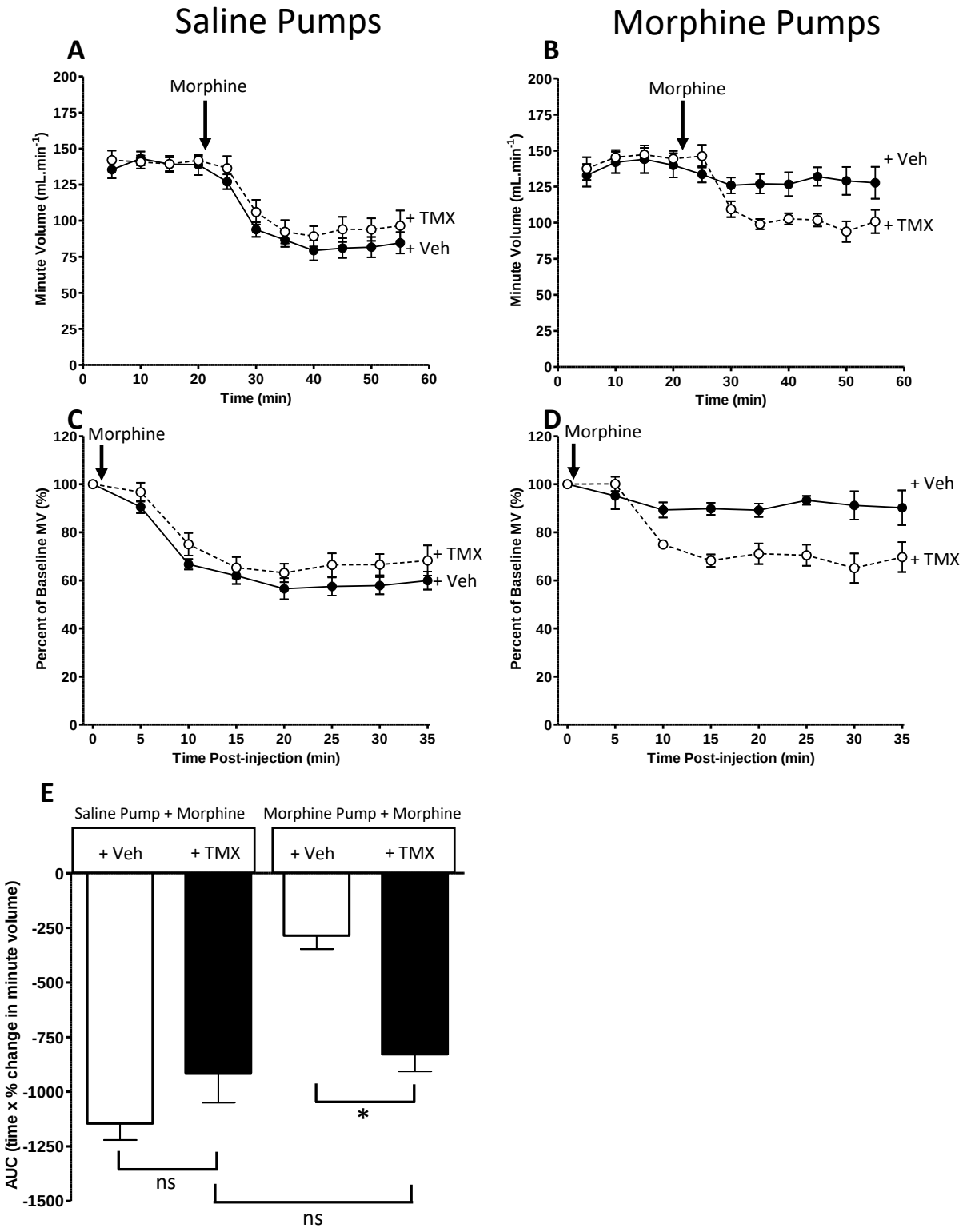


Figure 5.1: Effect of Tamoxifen Pre-treatment on tolerance to morphine respiratory depression. *(**A** & **C**) 6d saline pump implanted mice were injected with tamoxifen (TMX 0.6 mg/kg) or vehicle (veh) 30 min prior to an injection of morphine (10 mg/kg). TMX pre-treatment did not significantly alter the decrease in MV seen following morphine administration when compared to veh (**E**). (**B** & **D**) 6d morphine pump implanted mice were injected with TMX or veh 30 min prior to an injection of morphine. Pre-treatment with TMX significantly enhanced the decrease in MV seen following morphine administration compared to mice receiving veh and morphine (**E**). * indicates p<0.05. Groups compared in two-by-two factorial by Two-way ANOVA with Bonferroni's comparison. N=7 for all groups.*

5.2.2 Effect of calphostin C on Tolerance Induced by Prolonged Morphine Treatment

Male CD-1 mice received a prolonged treatment of either morphine or saline through implantation of an osmotic mini-pump for 6 d (See Materials and Methods section 2.4.1). Calphostin C (45 μg/kg) or vehicle (1% DMSO/99% saline) was administered 10 min prior to the recording of mouse respiration and a total of 30 min prior to the administration of an acute challenge dose of morphine (10 mg/kg).

Calphostin C administered to morphine pump-implanted mice prior to a saline challenge did not cause a decrease in MV (Fig. 5.2B, 5.2D-E). This data suggests that not only does calphostin C not depress MV inherently, but that the inhibition of PKC by calphostin C is insufficient to reverse tolerance to the circulating levels of morphine, provided for by the implanted pump.

An acute morphine challenge administered to saline pump-implanted mice caused a decrease in MV in both vehicle or calphostin C pre-treated groups (Fig. 5.2A & 5.2C). The decrease in MV was not significantly different between vehicle or calphostin C pre-treated mice (Fig. 5.2E). The same acute morphine challenge administered to morphine pump-implanted mice, and pre-treated with vehicle, did not cause a decrease in MV (Fig. 5.2B & 5.2D).

However, a morphine challenge administered to morphine pump-implanted mice, pre-treated with calphostin C, decreased MV significantly compared to vehicle control (Fig. 5.2B & 5.2D-E). This was not significantly different from the morphine-induced decrease in MV induced in saline pump implanted mice (Fig. 5.2E).

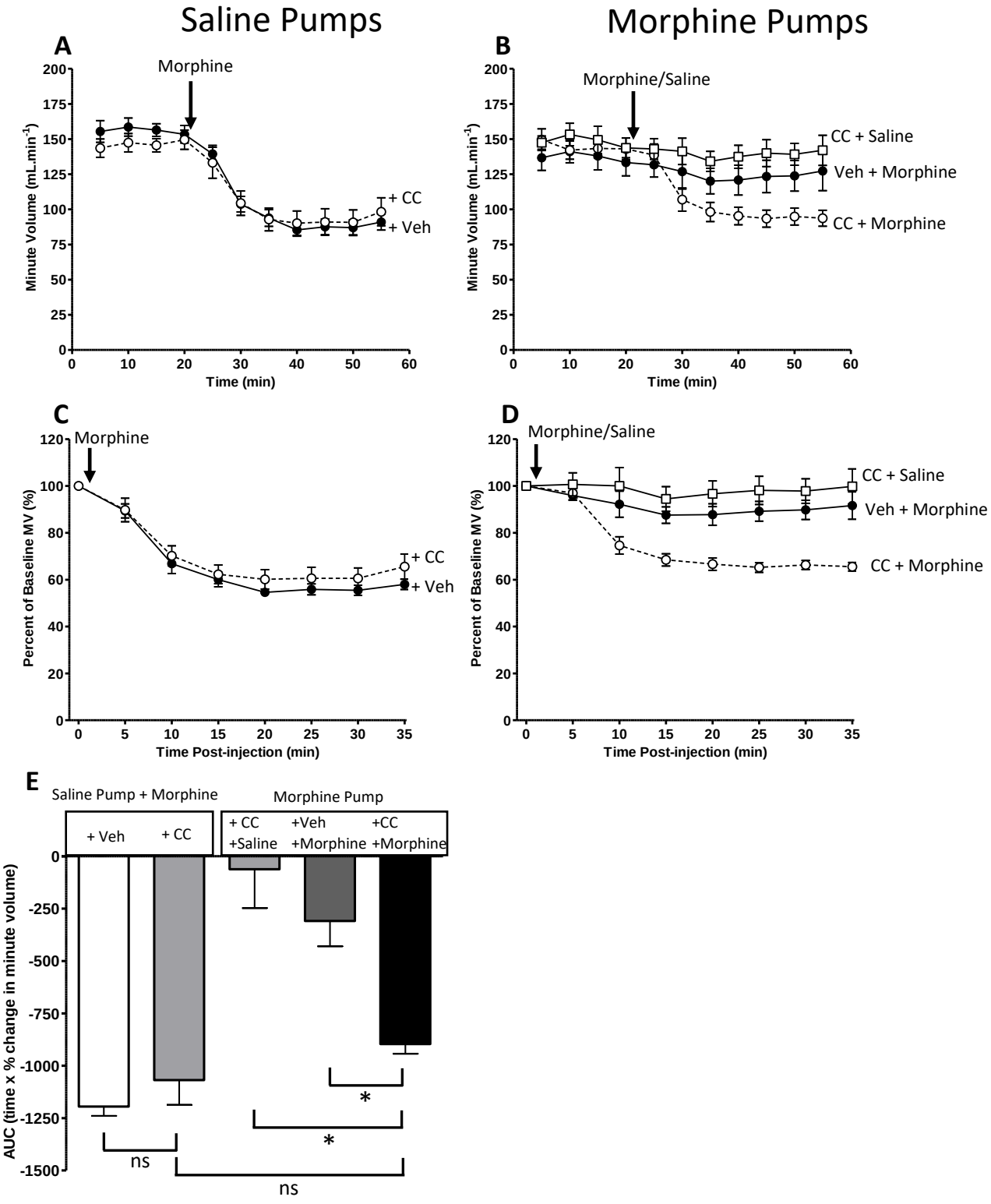


Figure 5.2: Effect of Calphostin C Pre-treatment on tolerance to morphine respiratory depression. *(**A** & **C**) 6d saline pump implanted mice were injected with calphostin C (CC 0.45 μg/kg) or vehicle (veh) 30 min prior to an injection of morphine (10 mg/kg). CC pre-treatment did not significantly alter the decrease in MV seen following morphine administration when compared to veh (**E**). (**B** & **D**) 6d morphine pump implanted mice were injected with CC or veh 30 min prior to an injection of morphine. Pre-treatment with CC significantly enhanced the decrease in MV seen following morphine administration compared to mice receiving veh and morphine (**E**). * indicates p<0.05. Groups compared in two-by-two factorial by Two-way ANOVA with Bonferroni's comparison. N=7 for all groups.*

5.2.3 Effect of Prolonged Morphine Treatment in PKCα Knock-out Mice

PKCα knock-out (KO) mice were procured from the laboratory of Professor Alistair Poole (University of Bristol), in order to ascertain the important of the PKCα subtype in the development of tolerance to morphine respiratory depression. Mixed sex PKCα KO mice and age matched wild-type (WT) littermates were implanted with morphine pumps for 6 d (See Materials and Methods section 2.4.1) before receiving an acute challenge of morphine (10 mg/kg) on day 6. Due to the limited availability of these mice, the control group of mice implanted with saline pumps for 6 d and receiving an acute challenge of morphine on day 6 was performed in C57/BL6 mice rather than littermate wild-type controls (shown in red in Fig. 5.3).

Despite their being a visually appreciable difference in the baseline respiration observed in WT mice compared to PKCα KO mice, this difference in baseline was not significantly different (Fig. 5.3D).

WT mice implanted with morphine pumps for 6 d did not display a significant decrease in MV following acute challenge with morphine on day 6. PKCα KO mice implanted with morphine pumps, on the other hand, had a significant decrease in MV compared to WT controls (Fig. 5.3A-C). The overall decrease in respiration seen following acute morphine administration in morphine pump implanted PKCα KO mouse was not significantly different from the decrease in MV seen following morphine administration in saline pump implanted C57/BL6 mice (Fig. 5.3C).

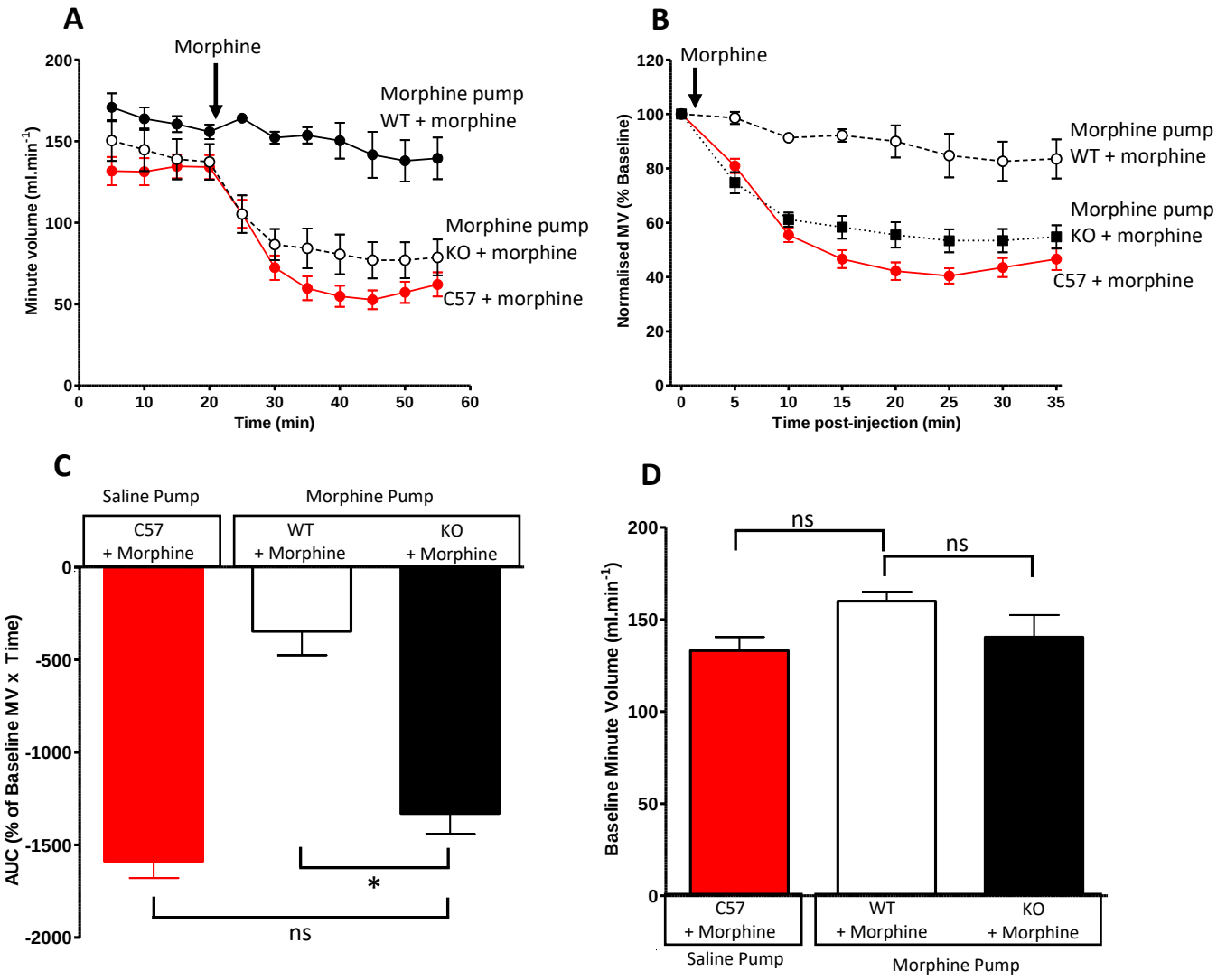


***Figure 5.3: Development of Tolerance to morphine respiratory depression in PKCα knock-out mice**. (**A** & **B**) Wild-type (WT) and knock-out (KO) mice were implanted with morphine pumps for 6d before being administered acute morphine (10 mg/kg). WT mice did not show a decrease in MV following morphine administration, whereas KO mice did. Background strain C57/BL6 mice were implanted with a saline pump for 6d before receiving acute morphine. Morphine administered to saline pump implanted mice caused a significant decrease in MV. (**C**) WT mice receiving prolonged morphine showed a significantly lower response to morphine compared to saline pump controls. KO mice did not show a significantly different response to saline pump controls. (**D**) The baseline levels of respiration were not significantly different across all three groups. * indicates p<0.05. Groups compared by One-way ANOVA and Bonferroni's comparison. N=7 for all groups.*

5.2.4 Effect of SP600125 on Tolerance Induced by Prolonged Morphine Treatment

Male CD-1 mice received a prolonged treatment of either morphine or saline through implantation of an osmotic mini-pump for 6d. SP600125 (20 mg/kg) or saline was administered 10 min prior to the recording of respiration and a total of 30 min prior to the administration of acute morphine (10 mg/kg) or saline, this pre-treatment period was determined from Melief et al (2010) to be sufficient for inhibition of JNK to occur.

Saline pump-implanted mice treated with SP600125 and challenged with saline did not display a change in MV compared to baseline, demonstrating that JNK inhibition by SP600125 did not depress MV (Fig. 5.4A-C). However, a separate group of saline pump-implanted mice treated with SP600125 and challenged with saline did display a significant increase in tail flick latency 30 min after administration of SP600125 (Fig. 5.4D). This is in agreement with Melief et al., (2010) and demonstrates that this dose of SP600125 was active in these assays.

Saline pump-implanted mice treated with saline and challenged with morphine saw a rapid and prolonged depression of MV (Fig. 5.4A-C). Morphine pump-implanted mice treated with SP600125 and challenged with morphine did not display a significant change in MV (Fig. 5.4A-C) and the overall decrease measured with AUC analysis of Fig. 5.4B was not significant from saline challenged and SP600125 treated controls (Fig. 5.4C).

A separate group of morphine pump-implanted mice treated with SP600125 and challenged with morphine did display a significant increase in tail flick latency 30 min after administration of SP600125, however this was not different from the saline pump-implanted and saline challenged controls treated with SP600125 (Fig. 5.4D)

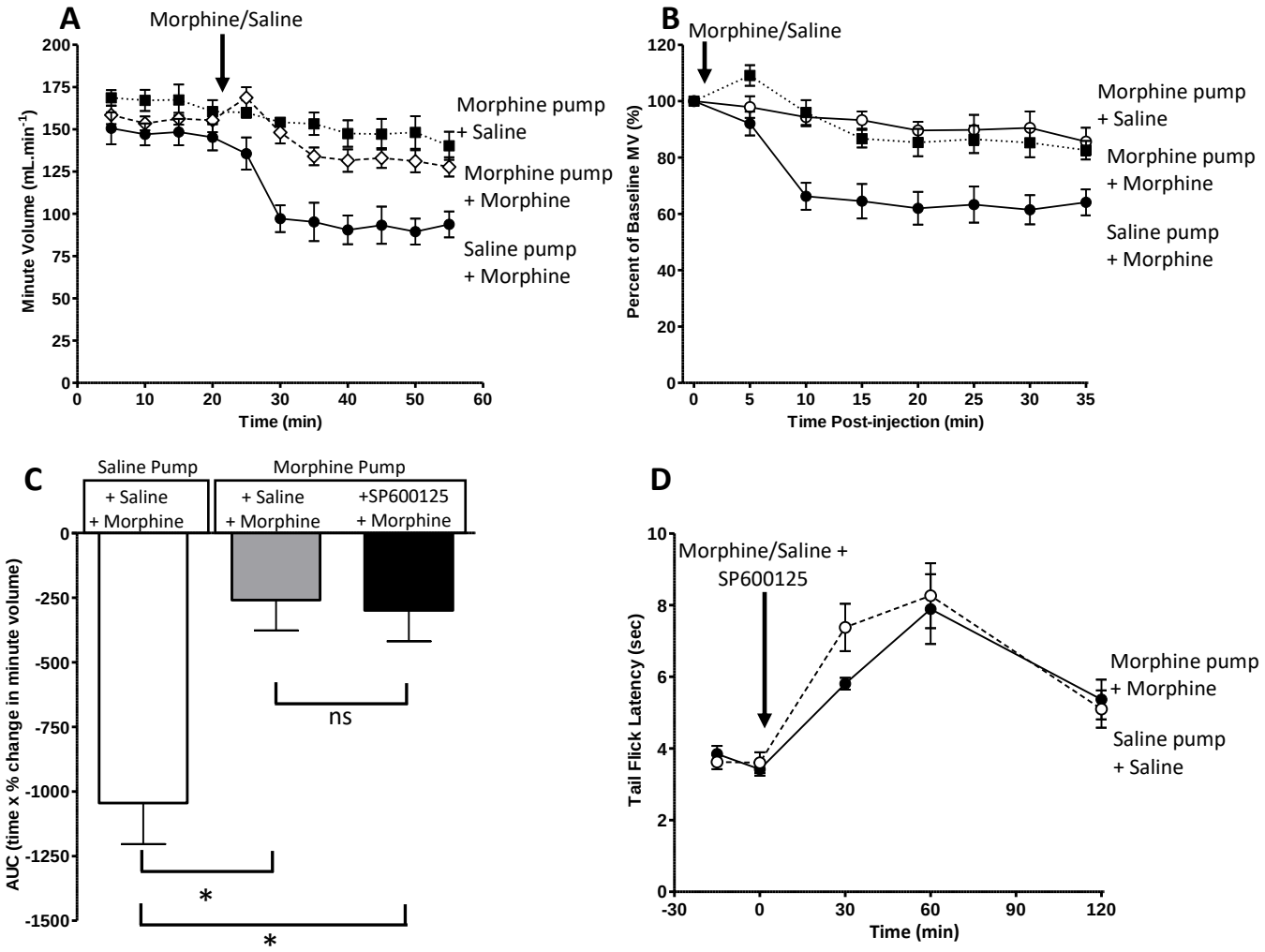


Figure 5.4: Effect of SP600125 Pre-treatment on tolerance to morphine respiratory depression and morphine antinociception*. (**A** & **B**) 6d saline pump implanted mice were injected with SP600125 (20 mg/kg) 30 min prior to an injection of morphine (10 mg/kg) which caused a large decrease in MV. 6d morphine pump implanted mice were injected with SP600125 (20 mg/kg) 30 min prior to an injection of morphine or saline. Neither saline or morphine administered after SP600125 caused a decrease in MV. (**C**) The response to both saline and morphine was significantly smaller in morphine pump implanted mice compared to the response to morphine in saline pump implanted mice. (**D**) Saline pump implanted mice co-administered saline and SP600125 showed a significant increase in mouse tail flick latency. Morphine pump implanted mice co-administered morphine and SP600125 also showed a significant increase in tail flick though this was not significantly different from saline control. * indicates p<0.05. In (**C**) groups were compared using a One-way ANOVA with Bonferroni's comparison. In (**D**) within group changes were compared using a Two-way repeated measures ANOVA with Bonferroni's comparison. Between group comparison were made using a Two-way ANOVA with Bonferroni's comparison. N=7 for all groups.*

5.3 Oxycodone

To investigate the potential mechanisms of oxycodone induced tolerance, different inhibitors of signalling pathways were administered to both medium and high oxycodone pump-implanted mice. These were not investigated in low oxycodone pump-implanted mice as the level of tolerance was considered to possess a low power of difference, thus requiring a significantly larger number of mice to confidently state the involvement or lack thereof of a given signalling mechanisms.

Therefore, the data shown in this section is exclusively from medium or high oxycodone pump-implanted experimental groups.

5.3.1 Effect of Calphostin C on Medium Oxycodone Induced Morphine Tolerance

Male CD-1 mice received a prolonged treatment of either medium oxycodone or saline through implantation of an osmotic mini-pump for 6d (See Materials and Methods section 2.4.1). Calphostin C (45 μg/kg) or vehicle (1% DMSO/99% saline) was administered 10 in prior to recording of mouse respiration and 30 min prior to the acute administration of morphine.

Calphostin C administered to medium oxycodone pump-implanted mice prior to a saline challenge did not cause a decrease in MV (Fig. 5.5B, 5.5D-E). This data suggests that not only does calphostin C not depress MV inherently, but that the inhibition of PKC by calphostin C is insufficient or unable to reverse tolerance to the circulating levels of oxycodone, provided for by the implanted pump

An acute morphine challenge administered to saline pump-implanted mice caused a decrease in MV in both vehicle or calphostin C pre-treated groups (Fig. 5.5A & 5.5C). The decrease in MV was not significantly different between vehicle or calphostin C pre-treated mice (Fig. 5.5E). The same acute morphine challenge administered to medium oxycodone pump-implanted mice, and pre-treated with vehicle, did not cause a decrease in MV (Fig. 5.5B & 5.5D).

However, a morphine challenge administered to medium oxycodone pump-implanted mice, pre-treated with calphostin C, decreased MV significantly compared to vehicle control (Fig. 5.5B & 5.5D-E). This was not significantly different from the morphine-induced decrease in MV induced in saline pump implanted mice (Fig. 5.5E).

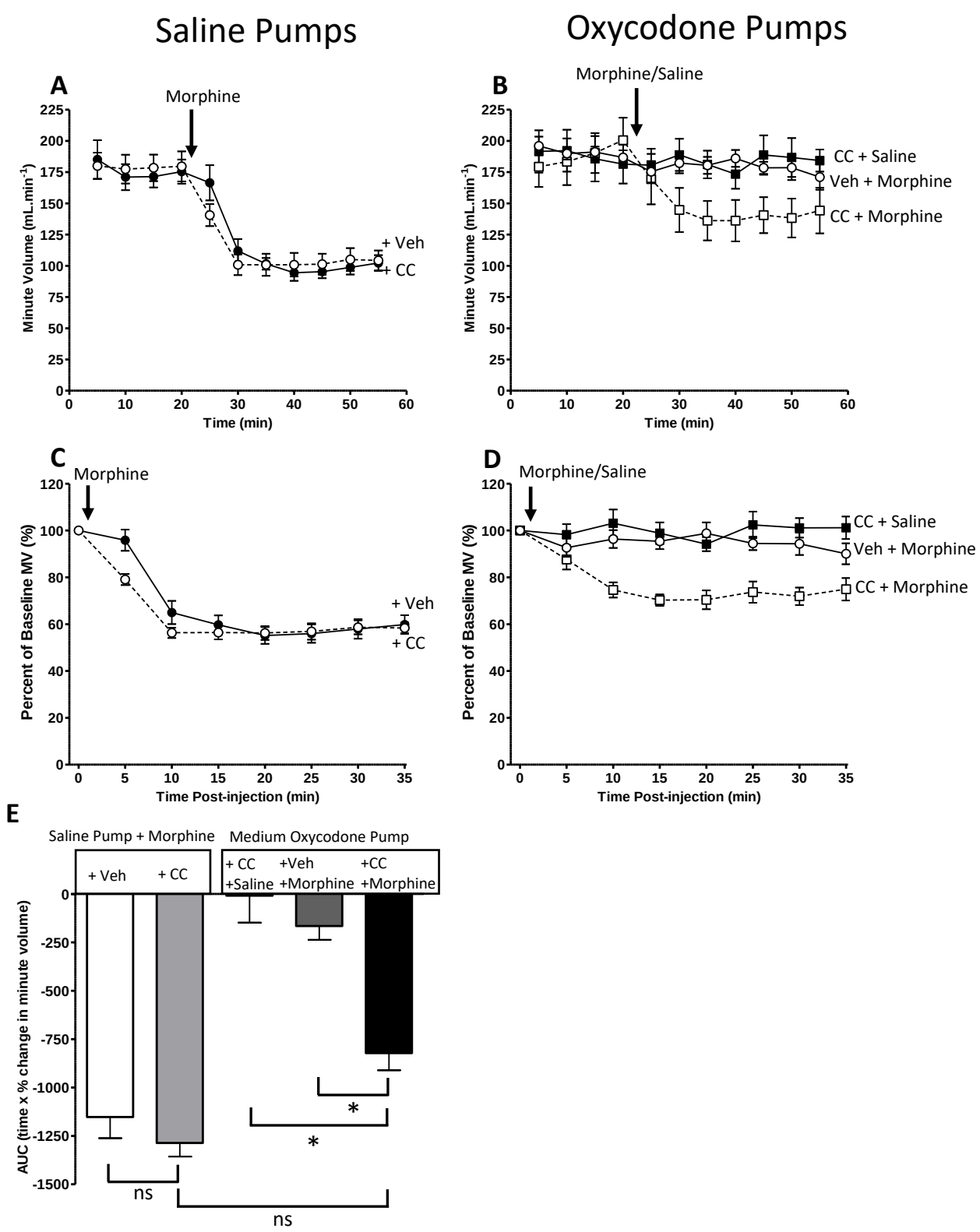


Figure 5.5: Effect of Calphostin C Pre-treatment on medium oxycodone treatment induced tolerance to morphine respiratory depression. *(**A** & **C**) 6d saline pump implanted mice were injected with calphostin C (CC 0.45 μg/kg) or vehicle (veh) 30 min prior to an injection of morphine (10 mg/kg). CC pre-treatment did not significantly alter the decrease in MV seen following morphine administration when compared to Veh (**E**). (**B** & **D**) 6d medium oxycodone pump implanted mice were injected with CC or veh 30 min prior to an injection of morphine. Pre-treatment with CC significantly enhanced the decrease in MV seen following morphine administration compared to mice receiving Veh and morphine (**E**). (**B**,**D**-**E**) 6d medium oxycodone pump implanted mice were injected with CC 30 min prior to an injection of saline. No change in MV was seen. * indicates p<0.05. Groups compared in two-by-two factorial by Two-way ANOVA with Bonferroni's comparison. N=7 for all groups.*

5.3.2 Effect of Calphostin C on High Oxycodone Induced Morphine Tolerance

Calphostin C (45 μg/kg) or vehicle (1% DMSO/99% saline) were also administered as a pre-treatment to mice implanted with high oxycodone pumps for 6d (See Materials and Methods section 2.4.1). Saline challenge after treatment with calphostin C did not decrease MV in high oxycodone pump-implanted mice (Fig. 5.6A & C). Additionally, morphine (10 mg/kg) challenge following vehicle treatment did not depress MV in high oxycodone pump-implanted mice (Fig. 5.6B & D).

Treatment with calphostin C prior to morphine challenge in high oxycodone pump-implanted mice did significantly depress MV compared to vehicle control (Fig. 5.6B & C). However, the decrease in MV following morphine and calphostin C pre-treatment in high oxycodone pump-implanted mice was still significantly reduced when compared to the depression of MV by morphine in saline pump-implanted and calphostin C treated controls (Fig. 5.6D).

Comparison of the degree of tolerance reversed by calphostin C treatment in medium and high oxycodone pump-implanted mice reveals that tolerance induced by a higher dose of oxycodone may be able to recruit a secondary mechanism of tolerance that is resistant to the inhibition of PKC.

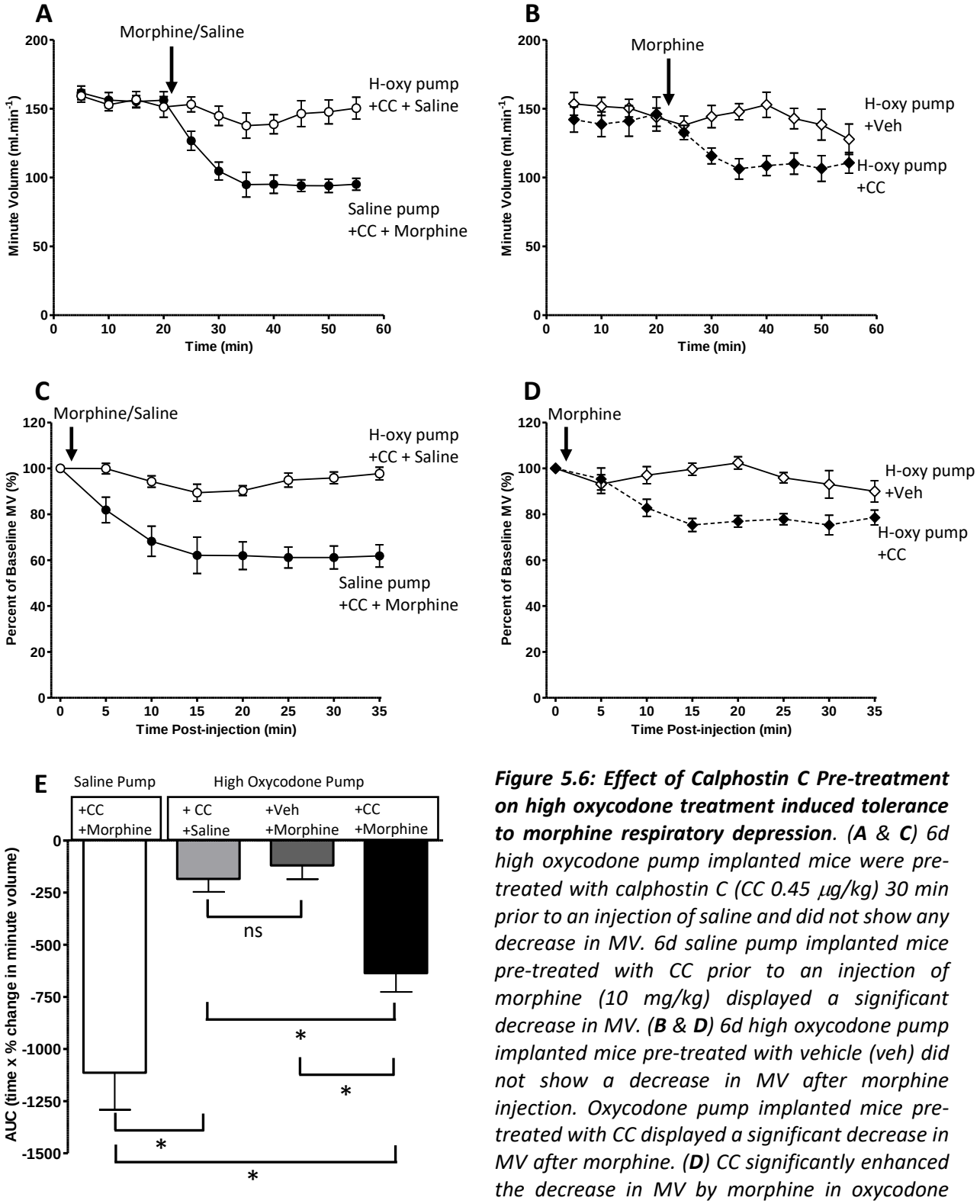


Figure 5.6: Effect of Calphostin C Pre-treatment on high oxycodone treatment induced tolerance to morphine respiratory depression*. (**A** & **C**) 6d high oxycodone pump implanted mice were pre-treated with calphostin C (CC 0.45 μg/kg) 30 min prior to an injection of saline and did not show any decrease in MV. 6d saline pump implanted mice pre-treated with CC prior to an injection of morphine (10 mg/kg) displayed a significant decrease in MV. (**B** & **D**) 6d high oxycodone pump implanted mice pre-treated with vehicle (veh) did not show a decrease in MV after morphine injection. Oxycodone pump implanted mice pre-treated with CC displayed a significant decrease in MV after morphine. (**D**) CC significantly enhanced the decrease in MV by morphine in oxycodone pump implanted mice compared to vehicle or saline control, however this response was still significantly reduced compared to saline pump implanted mice. * indicates p<0.05. Groups compared by One-way ANOVA with Bonferroni's comparison (**D**). N=7 for all groups.*

5.4 Methadone

5.4.1 Effect of Tamoxifen and Calphostin C on Prolonged Methadone Induced Morphine Tolerance

Previously published research as part of the authors MSc (Res) (See Appendix 1) demonstrated that acute ethanol was able to acutely reverse morphine induced tolerance but not methadone induced tolerance to morphine respiratory depression (Hill et al., 2016). These data suggest that methadone induces tolerance by a different signalling mechanism than morphine. Morphine tolerance is also reversible by PKC inhibition (See section 5.2), and so to examine whether the inability of ethanol to reverse methadone induced tolerance is due to morphine recruiting PKC and methadone recruiting a different mechanism, methadone pump implanted mice were treated with the PKC inhibitors calphostin C and tamoxifen.

Methadone pump-implanted mice challenged with morphine (10 mg/kg) after treatment with vehicle (10% propylene glycol/1% DMSO/89% saline) for 30 min did not see a depression of MV (Fig. 5.7A-D). Saline pump-implanted mice challenged with morphine after treatment with vehicle displayed a significant depression of MV (Fig. 5.7A-D). Morphine challenge did not depress MV in methadone pump-implanted mice treated with either tamoxifen (Fig. 5.7A, C & E) or calphostin C (Fig. 5.7B, D & E) for 30 min prior to the morphine challenge.

These data indicate that methadone tolerance is maintained by a signalling pathway that does not involve PKC. Therefore, the inability of ethanol to reverse methadone induced tolerance may be due to ethanol acting through a PKC dependent pathway, that methadone does not activate.

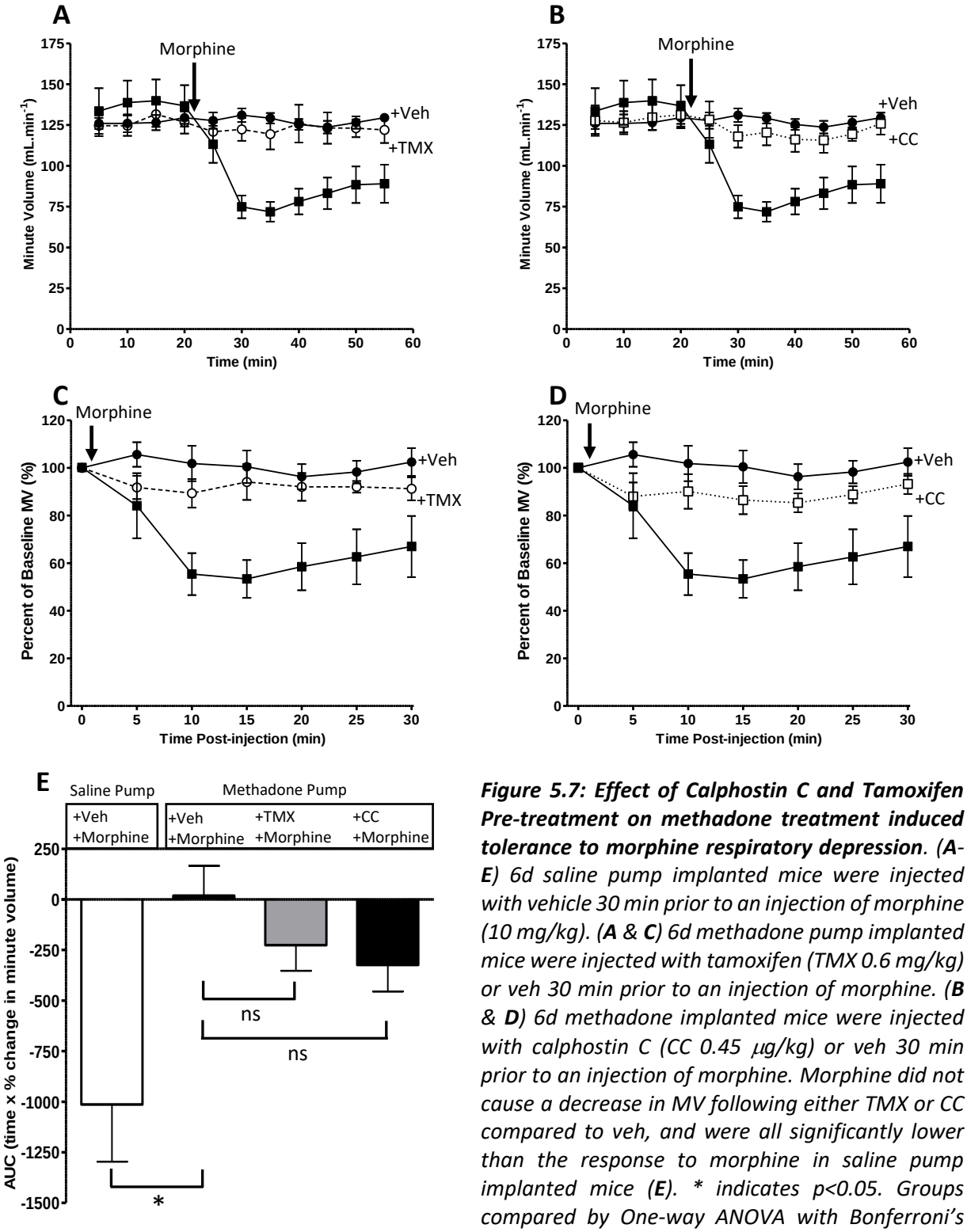


Figure 5.7: Effect of Calphostin C and Tamoxifen Pre-treatment on methadone treatment induced tolerance to morphine respiratory depression*. (**A**-**E**) 6d saline pump implanted mice were injected with vehicle 30 min prior to an injection of morphine (10 mg/kg). (**A** & **C**) 6d methadone pump implanted mice were injected with tamoxifen (TMX 0.6 mg/kg) or veh 30 min prior to an injection of morphine. (**B** & **D**) 6d methadone implanted mice were injected with calphostin C (CC 0.45 μg/kg) or veh 30 min prior to an injection of morphine. Morphine did not cause a decrease in MV following either TMX or CC compared to veh, and were all significantly lower than the response to morphine in saline pump implanted mice (**E**). * indicates p<0.05. Groups compared by One-way ANOVA with Bonferroni's comparison. N=7 for all groups.*

5.5 Fentanyl

Previous work has suggested that tolerance to fentanyl antinociception is primarily mediated by GRKs. Compound 101 (C101), a novel GRK2/3 inhibitor was used to investigate the role of GRk2/3 may play in the development of acute fentanyl antinociceptive tolerance. Calphostin C was also used to investigate any potential role PKC activation may play in the development of acute fentanyl antinociceptive tolerance.

5.5.1 Effect of Compound 101 on Acute Tolerance to Fentanyl Antinociception

Two groups of mice were used to investigate the role of GRK2/3 in acute fentanyl antinociceptive tolerance. Tolerance to fentanyl antinociception was induced by a two-dose injection protocol (See Materials and Methods section 2.4.2), with tail flick latency measured following administration of the first and second dose of fentanyl (0.15 mg/kg i.p.). 20 Min prior to administration of the second fentanyl dose, one group of mice were treated with vehicle and the other group of mice were treated with C101 (10 mg/kg i.p.).

A significant increase in mouse tail flick latency was measured in both groups of mice following the first dose of fentanyl (Fig. 5.8A-B). However, the second dose of fentanyl resulted in a smaller tail flick latency increase, in mice treated with vehicle prior to the second dose of fentanyl (Fig. 5.8A). In comparison, the second dose of fentanyl produced the same tail flick latency increase as the first dose of fentanyl in mice treated with C101 prior to the second dose of fentanyl (Fig. 5.8B).

Calculating the tail flick response following the first dose of fentanyl in Fig. 5.8 A&B as %MPE demonstrates that the antinociceptive response to the first dose of fentanyl was not significantly different between vehicle and C101 treated mice (Fig. 5.8C). Whereas the %MPE calculated following the second dose of fentanyl in Fig. 5.8 A&B is significantly greater in C101 treated mice compared to vehicle treated mice (Fig. 5.8D).

In addition to mice administered two doses of fentanyl, a single group of mice were administered a dose of saline followed by a single dose of fentanyl after treatment with C101. This group was used to assess the potential that GRK2/3 inhibition by C101 may enhance the acute antinociceptive effect of fentanyl. This may occur if rapid desensitization of the MOPr by fentanyl reduces the peak antinociceptive effect measured. However, area under the curve (AUC) analysis of the %MPE response following acute single fentanyl administration (Fig. 5.8E) in mice treated with C101, demonstrates that the fentanyl antinociception was not significantly different from controls (Fig. 5.8F).

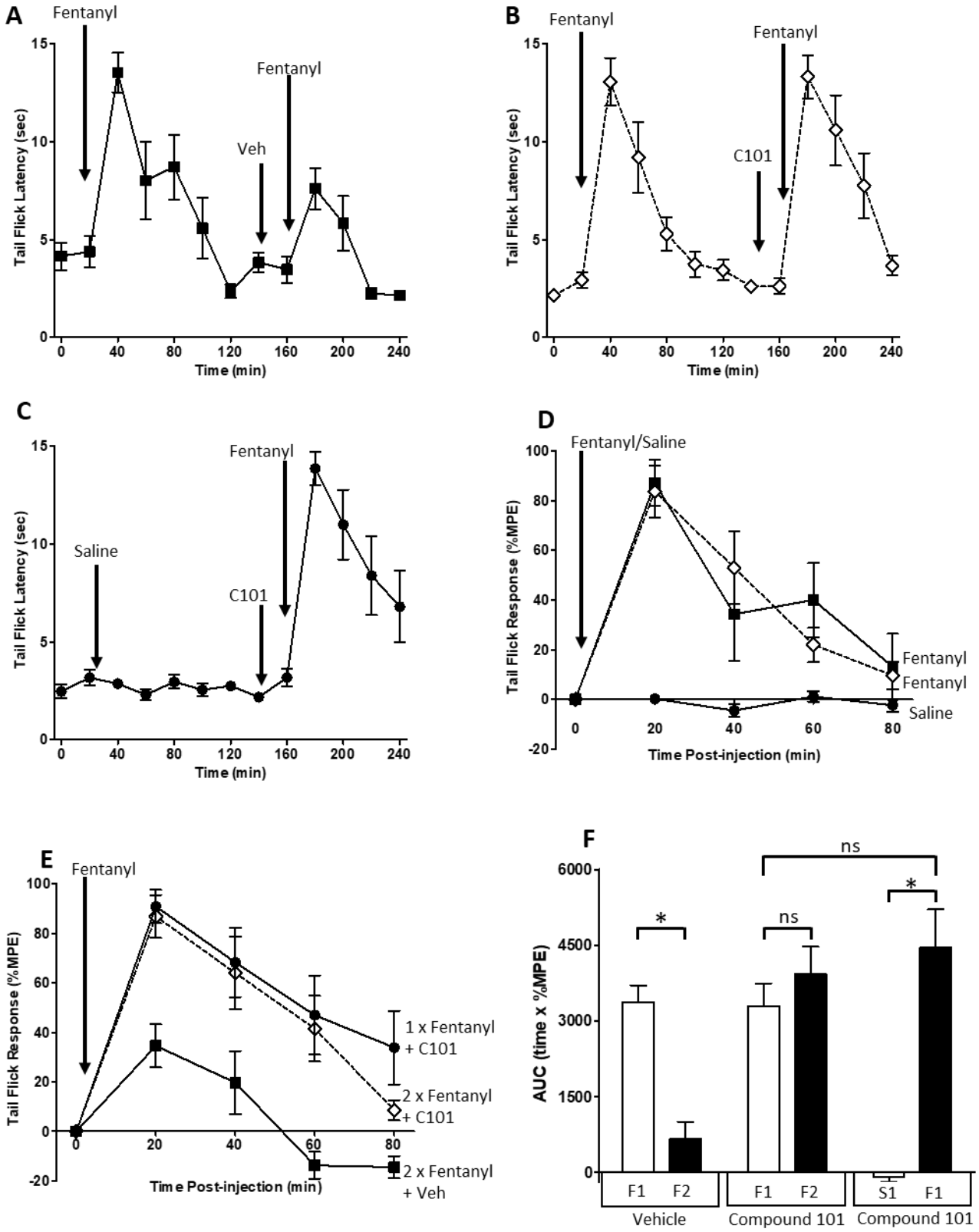


Figure 58: Effect of Compound 101 on acute tolerance to fentanyl antinociception*. (**A**) Fentanyl (0.15 mg/kg) induced increase in tail flick was reduced following the second injection of fentanyl when mice were pre-treated with vehicle (veh). (**B**) Pe-treatment with C101 prevented the reduced tail flick increase following the second injection of fentanyl. (**C**) Saline did not alter tail flick latency in mice. (**D**) The %MPE responses to the first injection of fentanyl were not different. (**E**) The %MPE following the second injection of fentanyl was not different in C101 pre-treated mice but was lower in veh treated mice. (**F**) Two injections of fentanyl (F1 & F2) results in a significantly decrease AUC following the second injection in veh pre-treated mice. The response to F2 in mice pre-treated with C101 was not significantly reduced compared to F1. C101 did not significantly enhance the response to fentanyl. Groups were compared using a Two-way ANOVA with Bonferroni's comparison. * indicates p<0.05. N=7-8 for each group.*

5.5.2 Effect of Compound 101 on Acute Tolerance to Fentanyl Respiratory Depression

All three groups of mice that had tail flick latency measured following fentanyl as displayed in Fig. 5.8, also had respiratory parameters measured prior to and following administration of each dose of fentanyl or saline.

Mice administered fentanyl (0.15 mg/kg i.p.) during the first dosing period displayed a reduction in minute volume, compared to no change in minute volume induced by saline (Fig. 5.9 A&C). Mice administered a second dose of fentanyl after vehicle treatment demonstrated a smaller reduction in MV when compared to the first dose of fentanyl (Fig 5.9 B, D&E).

However, whilst C101 treatment was able to prevent acute fentanyl antinociceptive tolerance (Fig. 5.8F), mice administered a second dose of fentanyl after C101 treatment also demonstrated a smaller reduction in MV when compared to the first dose of fentanyl (Fig 5.9 B, D&E).

The acute depression of mouse minute volume by acute fentanyl was not enhanced by treatment with C101 (Fig. 5.9E). These data demonstrate that inhibition of GRK2/3 is able to reverse the development of tolerance to acute fentanyl antinociception, but it is unable to reverse the development of acute tolerance to fentanyl respiratory depression,

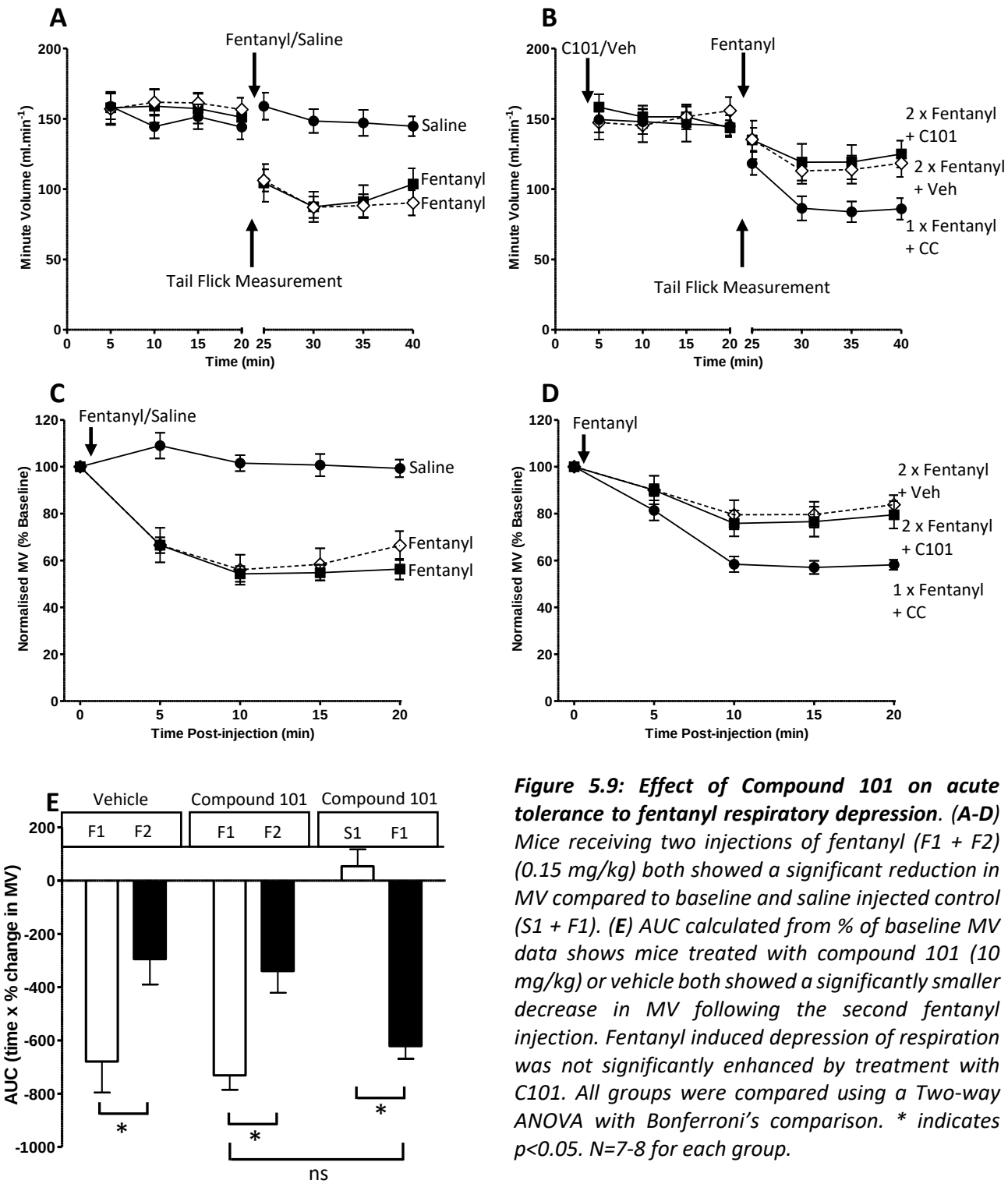


Figure 5.9: Effect of Compound 101 on acute tolerance to fentanyl respiratory depression*. (**A-D**) Mice receiving two injections of fentanyl (F1 + F2) (0.15 mg/kg) both showed a significant reduction in MV compared to baseline and saline injected control (S1 + F1). (**E**) AUC calculated from % of baseline MV data shows mice treated with compound 101 (10 mg/kg) or vehicle both showed a significantly smaller decrease in MV following the second fentanyl injection. Fentanyl induced depression of respiration was not significantly enhanced by treatment with C101. All groups were compared using a Two-way ANOVA with Bonferroni's comparison. * indicates p<0.05. N=7-8 for each group.*

5.5.3 Effect of Calphostin C on Acute Tolerance to Fentanyl Antinociception

Tolerance to fentanyl antinociception and fentanyl respiratory depression was induced in an additional group of mice to the C101 experiment. This group was used to investigate calphostin C, a PKC inhibitor, and its potential effect on acute fentanyl tolerance. The same vehicle was used to dissolve calphostin C as C101, and so the vehicle control group (Fig. 5.10A) is the same as shown in Fig. 5.8 and Fig. 5.9.

The first dose of fentanyl induced a significant increase in tail flick latency compared to baseline (Fig. 5.10 B). The second dose of fentanyl administered after treatment with calphostin C induced a smaller increase in tail flick latency. Calculating the tail flick response to each dose of fentanyl as %MPE from Fig. 5.10B, one can see that in the presence of calphostin C, significant tolerance to fentanyl antinociception occurs (Fig. 5.10E-F).

An additional group of mice were administered a dose of saline followed by a single dose of fentanyl after treatment with calphostin C. This group was used to assess the potential that PKC inhibition by calphostin may enhance the acute antinociceptive effect of fentanyl. However, area under the curve (AUC) analysis of the %MPE response following acute single fentanyl administration (Fig. 5.10E) in mice treated with calphostin C, demonstrates that the fentanyl antinociception was not significantly different from controls (Fig. 5.10F)

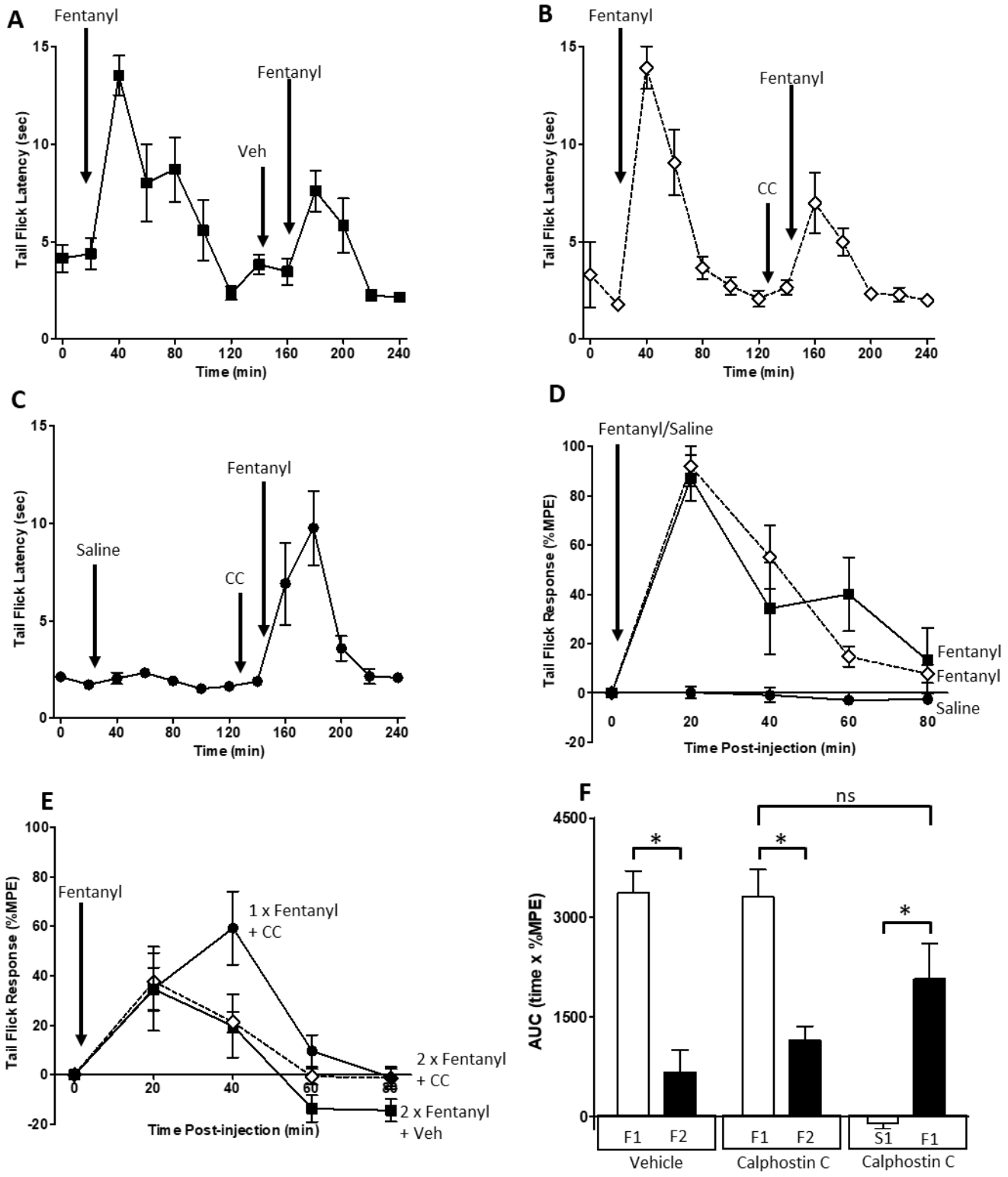


Figure 5.10: Effect of calphostin C on acute tolerance to fentanyl antinociception. *(**A**) Fentanyl (0.15 mg/kg) induced increase in tail flick was reduced following the second injection of fentanyl when mice were pre-treated with vehicle (veh). (**B**) Pe-treatment with calphostin C (CC 45 μg/kg) did not prevent the reduced tail flick increase following the second injection of fentanyl. (**C**) Saline did not alter tail flick latency in mice. (**D**) The %MPE responses to the first injection of fentanyl were not different. (**E**) CC significantly reduced the peak effect of fentanyl compared the response to first injection. (**F**) Two injections of fentanyl (F1 & F2) results in a significantly decrease AUC following the second injection in veh pre-treated mice. CC did not prevent the reduced response to F2. The overall response to fentanyl was not significantly reduced by CC. Groups were compared using a Two-way ANOVA with Bonferroni's comparison. * indicates p<0.05. N=7-8 for each group.*

5.5.4 Effect of Calphostin C on Acute Tolerance to Fentanyl Respiratory Depression

Fentanyl induced respiratory depression was measured alongside fentanyl induced antinociception in mice administered calphostin C and compared to vehicle treated mice.

The second dose of fentanyl in calphostin C treated mice produced less depression of mouse minute volume than the first administered dose of fentanyl (Fig. 5.11A-E). Additionally, calphostin C did not alter the acute depression of minute volume by fentanyl with the peak effect and overall effect remaining unchanged (Fig. 5.11C-E).

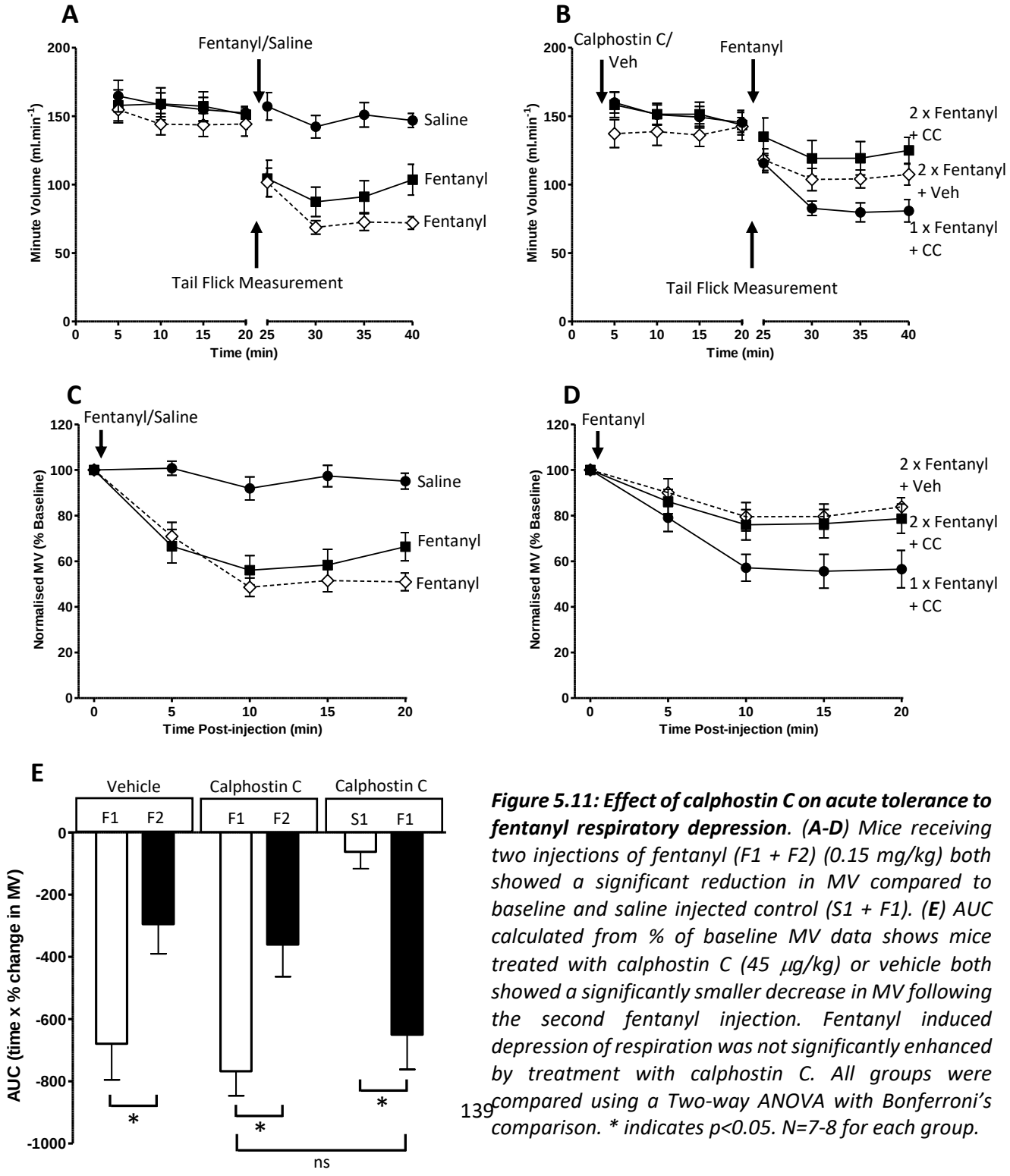


***Figure 5.11: Effect of calphostin C on acute tolerance to fentanyl respiratory depression**. (**A-D**) Mice receiving two injections of fentanyl (F1 + F2) (0.15 mg/kg) both showed a significant reduction in MV compared to baseline and saline injected control (S1 + F1). (**E**) AUC calculated from % of baseline MV data shows mice treated with calphostin C (45 μg/kg) or vehicle both showed a significantly smaller decrease in MV following the second fentanyl injection. Fentanyl induced depression of respiration was not significantly enhanced by treatment with calphostin C. All groups were compared using a Two-way ANOVA with Bonferroni's comparison. * indicates p<0.05. N=7-8 for each group.*

5.6 Discussion

5.6.1 Morphine

5.6.1.1 Protein Kinase C

Previous work has suggested that PKC, in particular PKCα, is a significant mediator of morphine desensitization of the MOPr and important in the maintenance of morphine tolerance *in vivo* (Bailey et al., 2009a, Bailey et al., 2009b, Hull et al., 2010, Lin et al., 2012). Tamoxifen has been identified as a putative PKC inhibitor in addition to its on target antagonism of oestrogen receptors (Xie et al., 2015, Yildiz et al., 2008, O'Brian et al., 1985), whereas calphostin C is known to inhibit PKC (Hermenegildo et al., 1993, Blazquez et al., 2018). Both tamoxifen and calphostin C were used as broad-spectrum PKC inhibitors targeting many isoforms of PKC.

These drugs are not as selective as other PKC inhibitors such as GF109203X and Go6976, but they are known to cross the blood brain barrier (Hermenegildo et al., 1993, O'Brian et al., 1985, Xie et al., 2015, Yildiz et al., 2008, Blazquez et al., 2018). This allows respiratory parameters to be assessed accurately without the need for ICV administration of PKC inhibitors.

Both tamoxifen and calphostin C were able to reverse tolerance to morphine induced respiratory depression, induced by prolonged morphine treatment. Whilst tamoxifen does antagonise oestrogen receptors, previous work within the laboratory has demonstrated that morphine tolerance is unaffected by the administration of an alternative oestrogen receptor antagonist (G1) (Withey et al., 2017). Additionally, the only overlap in kinase inhibition between tamoxifen and calphostin C is the inhibition of PKC, making the confluence of their effects likely to be through PKC inhibition rather than an effect on other signalling transducers.

The administration of either tamoxifen or calphostin C alone was not sufficient to induce respiratory depression in morphine tolerant mice. This is somewhat perplexing given that the morphine pump utilised in these experiments is known to produce a significant brain and blood concentration of morphine on day 6 of the experiment (See Fig. 6.7). One might assume that with significant circulating morphine, inhibition of PKC would reverse morphine tolerance at the MOPr and thus allow circulating morphine to activate MOPrs and produce a depression of respiration. Given that this is not seen, and reversal of tolerance is only observed when an additional dose of morphine is administered following either tamoxifen or calphostin C treatment, it may be possible that the morphine present in the brain on day 6 of pump implantation is simply not freely available to bind to the MOPr. This may be due to morphine re-distribution to lipid.

In addition to the use of broad spectrum PKC inhibitors to investigate morphine tolerance, PKCα KO mice were studied. Implantation of morphine pumps for 6 days was unable to produce tolerance in PKCα KO mice, whilst tolerance was readily observed in WT littermates. Once again this suggests a crucial role for PKC, in particular PKCα in morphine tolerance. Considering the data from acute inhibition of PKC and complete absence of PKCα both reversing and preventing tolerance respectively, it would strongly suggest that for morphine at least, PKC is a vital component in both the establishment and maintenance of tolerance at the MOPr.

There have been several suggestions of putative phosphorylation sites on the third intracellular loop and C-tail of the MOPr (Feng et al., 2011, Law and Loh, 1999, Zhang et al., 1996, Mousa et al., 2016) that PKC may directly phosphorylate to induce changes in the activity of the MOPr. Phosphorylation of the MOPr is known to occur following activation by opioid agonists (Schulz et al., 2004, Williams et al., 2013) and point mutations of phosphorylation sites are known to prevent MOPr desensitization by morphine (Schulz et al., 2004).

These data, coupled with PKC inhibition preventing agonist specific desensitization (Bailey et al., 2009a, Bailey et al., 2009b) collectively suggest that PKC plays a vital role in the development of tolerance to some MOPr agonists. This is likely to require some degree of interaction and phosphorylation of the MOPr, though it is not currently known whether PKC does this directly or indirectly (Williams et al., 2013).

5.6.1.2 c-Jun N-terminal Kinase

Contrary to previously published work suggesting that JNK signalling played an important part in morphine tolerance (Melief et al., 2010), pre-treatment with the broad -spectrum JNK inhibitor SP600125, was not found to affect tolerance to morphine respiratory depression following prolonged morphine treatment. However, the previously published work by Melief et al utilised a JNK2 KO mutant mouse and thus JNK was impaired throughout development and during every exposure to morphine in these mice. JNK may potentially play a role in the induction of morphine tolerance, but once tolerance has been established, as on day 6 of these experiments, JNK inhibition may not be sufficient to alter the expression of tolerance.

5.6.1.3 Conclusion

These data support previous work that has identified PKC as a key mediator of morphine tolerance. Acute inhibition of PKC was able to reverse established morphine tolerance, suggesting that PKC is vital for the ongoing maintenance of tolerance at the level of the MOPr. Similarly, PKCα mice were not able to develop tolerance to morphine, suggesting that PKCα at least, is crucial in the formative stages of morphine tolerance. Whilst inhibition of JNK did not alter established morphine tolerance, this may be due to JNK signalling being important at earlier time points during the development of morphine tolerance.

It should also be noted that there is evidence suggesting that morphine recruitment of GRK is a major mechanism by which morphine induces tolerance (Gluck et al., 2014). However, morphine is considered to have relatively low intrinsic efficacy for the recruitment of GRK compared to more potent MOPr agonists such as DAMGO (McPherson et al., 2010). Considering that several publications have demonstrated the divergence of desensitization and tolerance mechanisms induced by morphine and DAMGO (Hull et al., 2010, Bailey et al., 2009b, Johnson et al., 2006), it seems unlikely that morphine induced GRK recruitment to the MOPr plays a significant role in morphine induced tolerance, otherwise one might expect to see more commonalities between DAMGO and morphine induced desensitization mechanisms.

5.6.2 Oxycodone

There is relatively little literature on the kinase mechanisms related to the development of oxycodone tolerance. A single publication has identified PKC as a likely mediator of oxycodone tolerance with PKC inhibition preventing oxycodone cellular tolerance in dorsal root ganglion neurones *in vitro* (Jacob et al., 2017). On the other hand, another single publication has tentatively suggested a role for GRK in the development of acute oxycodone tolerance, with oxycodone having a decreased profile for internalisation following GRK inhibition in HEK293 cells (Melief et al., 2010).

Characterisation of a plethora of opioid agonist by Mcpherson et al (2010) demonstrated that oxycodone has a similar signalling profile to that of morphine, when considering relative intrinsic efficacy for G-protein dependent and GRK/arrestin dependent signalling pathways. It was therefore considered a sound hypothesis that oxycodone might recruit a similar kinase system for inducing tolerance as that by morphine.

Utilising the medium oxycodone induction of tolerance, it is clear from these data that PKC inhibition can reverse tolerance induced to morphine respiratory depression to a similar degree seen with morphine induced morphine tolerance. However, increasing the dose of oxycodone appears to result in a degree of tolerance that is unaffected by the inhibition of PKC. Given that the existing literature suggests both PKC and GRK as mechanisms for the induction of oxycodone tolerance, it may be that PKC is recruited preferentially at lower doses of oxycodone during tolerance induction, but GRK recruitment occurs only at higher doses.

Due to a scarcity of the GRK inhibitor compound 101, administering both calphostin C and compound 101 to inhibit PKC and GRK simultaneously in high oxycodone pump implanted mice was not possible. However, this is considered an important experiment required as investigation into oxycodone induced tolerance moves forwards.

5.6.3 Methadone

Previously published work has reported that methadone desensitization at the MOPr in brain slices taken from mice treated for 6d with methadone pumps (Quillinan et al., 2011). Quillinan et al describe methadone induced desensitization to met-enkaphalin at the MOPr as being unaffected by GRK2 inhibition.

Given the reported data suggesting methadone desensitization, as a proxy for tolerance induced in vivo, was unaffected by GRK2 inhibition, both tamoxifen and calphostin C were used to investigate a potential role for PKC activation in methadone induced tolerance to morphine. However, the data clearly demonstrates that PKC inhibition does not alter established tolerance to morphine respiratory tolerance when induced by prolonged methadone.

Unfortunately, the scarcity of compound 101 once again prevented further investigation into the role of GRK2/3 in methadone induced tolerance. Further work would consider the inhibition of GRK2/3 by compound 101 a vital and necessary experiment to conduct. Additionally, there may potentially be an involvement of JNK in methadone induced tolerance, though this has not been suggested within the literature as a mechanism of methadone tolerance, given that multiple other opioids have been suggested to function through JNK signalling, it places JNK has a high priority target for investigation.

5.6.4 Fentanyl

Acute tolerance to fentanyl antinociception has previously been reported (Melief et al., 2010). Melief et al additionally reported that they were unable to induce acute tolerance to fentanyl in GRK2 KO mice, suggesting that GRK2 was crucial for the development of tolerance to fentanyl, at least acutely. More recently, published work has described fentanyl as an GRK/arrestin biased opioid agonist (Schmid et al., 2017a), further strengthening the evidence that fentanyl primarily signals through this pathway. These data align with the signalling profile for fentanyl reported in Mcpherson et al, demonstrating fentanyl has a relatively high degree of intrinsic efficacy for arrestin signalling.

Administration of compound 101 to inhibit GRK2/3 was very clearly able to prevent the development of acute tolerance to fentanyl antinociception. However, compound 101 administration was unable to affect the development of tolerance to fentanyl induced respiratory depression. The pharmacokinetics of compound 101 have been poorly characterised, and it is possible that whilst being able to penetrate and inhibit GRK within the lower spinal column, utilised in the tail flick spinal reflex, compound 101 was unable to fully inhibit GRK within the central brain stem nuclei responsible for respiratory control. It is equally possible however, that fentanyl antinociception and fentanyl respiratory depression were mediated by distinct mechanisms. This may be due to relative expression of signalling proteins within different brain and spinal regions, which predisposes a given signalling pathway to be dominant, when insufficient protein exists to mediate the other. It has certainly suggested that nucleus specific expression of RGS proteins may play role in G-protein signalling (Gold et al., 1997).

As tolerance to fentanyl respiratory depression may have been mediated by a distinct kinase pathway to GRK, inhibition of PKC by calphostin C was used to investigate a potential role for PKC in tolerance to fentanyl respiratory depression. Given that PKC inhibition did not result in a loss of tolerance to fentanyl respiratory depression, it seems safe to conclude that PKC is not involved. Calphostin C, as evidenced by multiple other experiments within this chapter and previous publications, clearly achieves brain penetrance. Though potentially calphostin C is unable to inhibit the specific isoform that fentanyl may signal via.

To further investigate the role of GRK in the development of tolerance to fentanyl respiratory depression, an improved GRK inhibitor with better pharmacokinetics and pharmaco-availability would greatly improve the clarity of these data. The Tesmer lab, responsible for the development of compound 101, has more recently developed a more specific GRK2/3 inhibitor known as 14aS. 14aS Has improved pharmacokinetics and bio-availability compared to C101 and so would be a better pharmacological tool to investigate the role of GRK2/3 in opioid tolerance (Waldschmidt et al., 2017).

There is no scientific consensus on the mechanism of opioid induced respiratory depression. However, the current dominant theory, is that opioid recruitment of GRK to the MOPr is responsible for opioid depression of respiration. This theory stems from a highly influential paper demonstrating reduced respiratory depression by morphine in arrestin-3 KO mice (Raehal et al., 2005). This has led to significant resources being directed in the pursuit of G-protein biased opioid agonists to circumvent the mechanisms considered responsible for the respiratory depressant effect of opioid (Schmid et al., 2017a, Manglik et al., 2016a, DeWire et al., 2013a).

One would therefore hypothesise that if fentanyl produced its respiratory depressant effect through an arrestin and by extension GRK dependent mechanisms, then inhibition of GRK would reduce the ability of fentanyl to depress respiration. However, inhibition of GRK2/3 by compound 101 did not decrease fentanyl respiratory depression. These data illustrating that GRK2/3 is not involved in fentanyl depression of respiration.

5.6.5 Overall Conclusion

These data appear to show a difference in the recruitment of G-protein versus GRK/arrestin signalling dependent on the relative efficacy of the opioid agonist. Morphine and oxycodone are relatively low efficacy agonists and appear to induce tolerance through primarily G-protein activation of PKC; whereas methadone and fentanyl are far higher efficacy agonists and a such PKC inhibition does not alter the induction of tolerance, yet GRK inhibition prevents fentanyl tolerance. This would suggest higher efficacy agonists are more likely to induce tolerance through GRK/arrestin recruitment.

Given the lack of published research on the specificity of compound 101 to inhibit GRK2/3 over GRK4/5/6 outside of isolated enzyme preparations, it is difficult to conclude that the effect of compound 101 is exclusively due to GRK2/3 inhibition. Additionally, there is no data to draw on regarding what percentage of inhibition of GRK2/3 activity is achieved at the doses of compound 101 used.

Indeed, whilst utilising better GRK inhibitors is a necessity to further our understanding of the role GRK has in the induction of opioid tolerance, simply an improved characterisation and in vivo profiling of existing inhibitors would also improve our knowledge surrounding the role of GRK in opioid tolerance.

6.0 Opioid Tolerance and Polydrug Abuse

6.1 Introduction

Opioids users commonly abuse multiple drugs concomitantly (Darke, 2003, Hickman et al., 2008b, White and Irvine, 1999). The abuse of multiple drugs can be desired for multiple reasons; this may be to achieve different results of intoxication from each individual drug, or it may be to enhance the effect of one drug by the addition of another. Whatever reason the mixture is consumed for, it is well understood that polydrug abuse represents a serious risk factor by increasing the chances of accidental opioid overdose (Darke, 2003, Hickman et al., 2008b, White and Irvine, 1999). Two drugs known to be co-abused with opioids are ethanol and pregabalin.

6.1.1 Ethanol

Ethanol is the most commonly detected drug in opioid overdose post-mortem analysis (Darke, 2003) with approximately 35% of all opioid overdose fatalities showing detectable levels of blood alcohol content (BAC measured as ethanol). However, in cases of heroin overdose with detectable BAC, the blood morphine content is paradoxically much lower than expected, and inversely correlated to BAC (Darke and Hall, 1995, Ruttenber et al., 1990). Indeed, the blood morphine content of living users is consistently as high or higher than that seen in heroin overdose deaths (Darke et al., 2002a, Monforte, 1977, Brewer, 2002). Additionally, BAC detected in heroin overdose death tends to be seen in experienced, long-term heroin users who would be expected to have considerable tolerance to opioid respiratory depression (Hall and Darke, 1998, Warner-Smith et al., 2001). This combination of lower than expected heroin levels with concomitant detection of blood ethanol in experienced heroin user's fatal overdoses, suggests an additional pharmacological interaction between ethanol and heroin use, beyond simple additive depression of the CNS by ethanol and heroin.

Acute administration of ethanol to mice is able to reverse morphine antinociceptive tolerance and return sensitivity to an acute dose of morphine (Hull et al., 2013). Ethanol has also been shown to reverse oxycodone tolerance when administered acutely to oxycodone tolerant mice (Jacob et al., 2017), yet ethanol has no effect on tolerance induced by methadone (Hill et al., 2015). Ethanol has also been shown to reverse desensitization of the MOPr following bath application of ethanol (20 mM) to brain slices that have either been acutely desensitized with morphine or are brain slices taken from rats that have received prolonged morphine treatment *in vivo* (Llorente et al., 2013).

These data suggest a fundamental effect of ethanol on signaling through the MOPr, dependent on the opioid agonist used to induced either MOPr desensitization or tolerance *in vivo*. The implication from being that ethanol interacts with a single or small subsection of opioid induced mechanisms of tolerance or desensitization at the MOPr.

6.1.2 Acetaldehyde

Acetaldehyde is the primary metabolite of ethanol. Previously acetaldehyde was thought to be exclusively aversive in its action ; indeed, a common treatment to aid recovering alcoholics was (and is) disulfiram, an inhibitor of acetaldehyde dehydrogenase (Peana and Acquas, 2013). Disulfiram prevents the metabolism of acetaldehyde leading to its accumulation, producing a strong aversive reaction in patients consuming alcohol (Brewer et al., 2017).

However, recent research has suggested that the aversive properties of acetaldehyde accumulation are mediated only through peripheral accumulation of acetaldehyde, rather activity of acetaldehyde in the CNS (Quertemont et al., 2005, Font et al., 2006, Font et al., 2013). The historical understanding of ethanol metabolism precluded acetaldehyde forming a significant concentration in the brain, due to the finding that ethanol could only be metabolized in the liver (Quertemont et al., 2004). However, the discovery that ethanol could be metabolized in situ by Brain Catalase (a common enzyme catalysing the conversion of hydrogen peroxide to water) and produce significant brain concentrations of acetaldehyde has shown this to be false (Correa et al., 2008).

Acetaldehyde has since been shown to produce its own rewarding effect and rodents treated with the acetaldehyde chelator d-penicillamine, decreased the rewarding properties of ethanol (Font et al., 2006). D-penicillamine does not affect the initial metabolic pathway of ethanol, nor its pharmacological activity, thus the reduction in reward could only come from the decrease in active metabolite through its chelate inactivation.

It must, therefore, be considered that acetaldehyde may in fact be a mediator of ethanol-opioid interactions. This was investigated with manipulation of acetaldehyde concentrations with d-penicillamine treatment, as well as direct administration of acetaldehyde.

6.1.3 Pregabalin

Pregabalin was originally prescribed as an anti-epileptic, however recent years have seen a surge in pregabalin prescription for other conditions, such as anxiety, pain, insomnia and bipolar disorder. These conditions are more common than epilepsy and thus there has been a considerable rise in the overall number of prescriptions written for pregabalin and the general prevalence of pregabalin in the homes of the public (Gomes et al., 2017).

Previously, this has not been considered notable due the identified 'low-abuse liability' of pregabalin; yet recent years have seen a clear indication that abuse of pregabalin in isolation does indeed occur (Mersfelder and Nichols, 2016, Schjerning et al., 2016), and yet more worrisome is evidence that pregabalin is co-abused with illicit drugs such as heroin (Lyndon et al., 2017).

Heroin overdose involving pregabalin has risen across Europe in recent years (Hakkinen et al., 2014). This surge in heroin and pregabalin based deaths has led to the current investigation into potential interaction between pregabalin and heroin tolerance. Hypothetically, pregabalin may function akin to ethanol (as discussed above) in returning sensitivity to an opioid agonist, acting at a previously desensitized MOPr or within an opioid tolerant system.

6.1.4 Chapter Aims

The aims of this chapter were:

i) To investigate the effect of acute low dose ethanol administration on morphine, medium oxycodone and high oxycodone induced tolerance.

ii) To investigate the effect of prolonged ethanol diet administration on the development of morphine tolerance.

iii) To investigate the effect of acute acetaldehyde administration, the primary metabolite of ethanol, on morphine tolerance.

iv) To investigate the effect of acute pregabalin administration on morphine, medium oxycodone, high oxycodone and methadone induced tolerance.

v) To assess the contribution of ethanol and pregabalin as depressant substances (at sub-depressant doses) and their contribution to the re-sensitization of the MOPr in addition to their known system depressant effects.

6.2 Acute Ethanol

6.2.1 Effect of Acute Ethanol on Morphine Induced Tolerance to Morphine Respiratory Depression

Male CD-1 mice received a prolonged treatment of either morphine or saline through implantation of an osmotic mini-pump for 6 d (See Materials and Methods section 2.4.1). Saline pump-implanted mice were co-administered a morphine challenge (10 mg/kg i.p.) with either saline or ethanol (0.3 g/kg i.p.) (Fig. 6.1A-B). Morphine depressed minute volume and the depression of minute volume by morphine was not enhanced or decreased when co-administered with ethanol compared to saline control (Fig. 6.1A-B).

An ethanol challenge, co-administered with saline in morphine pump-implanted mice did not depress minute volume (Fig. 6.1C-D). Similarly, a morphine challenge, co-administered with saline in morphine pump-implanted mice did not depress minute volume (Fig. 6.1E-F) and was significantly decreased compared to the effect of morphine administered to saline pump-implanted mice. However, co-administration of a morphine challenge with ethanol in morphine pump-implanted mice did cause a significant depression of minute volume (Fig. 6.1E-G).

Area under the curve (AUC) values were calculated from figures 6.1B, 6.1D and 6.1F and plotted in figure 6.1G. The depression of minute volume induced by a morphine challenge co-administered with ethanol in morphine pump-implanted mice was not significantly different from the depression of minute volume seen in saline pump implanted mice co-administered morphine and ethanol (Fig. 6.1G).

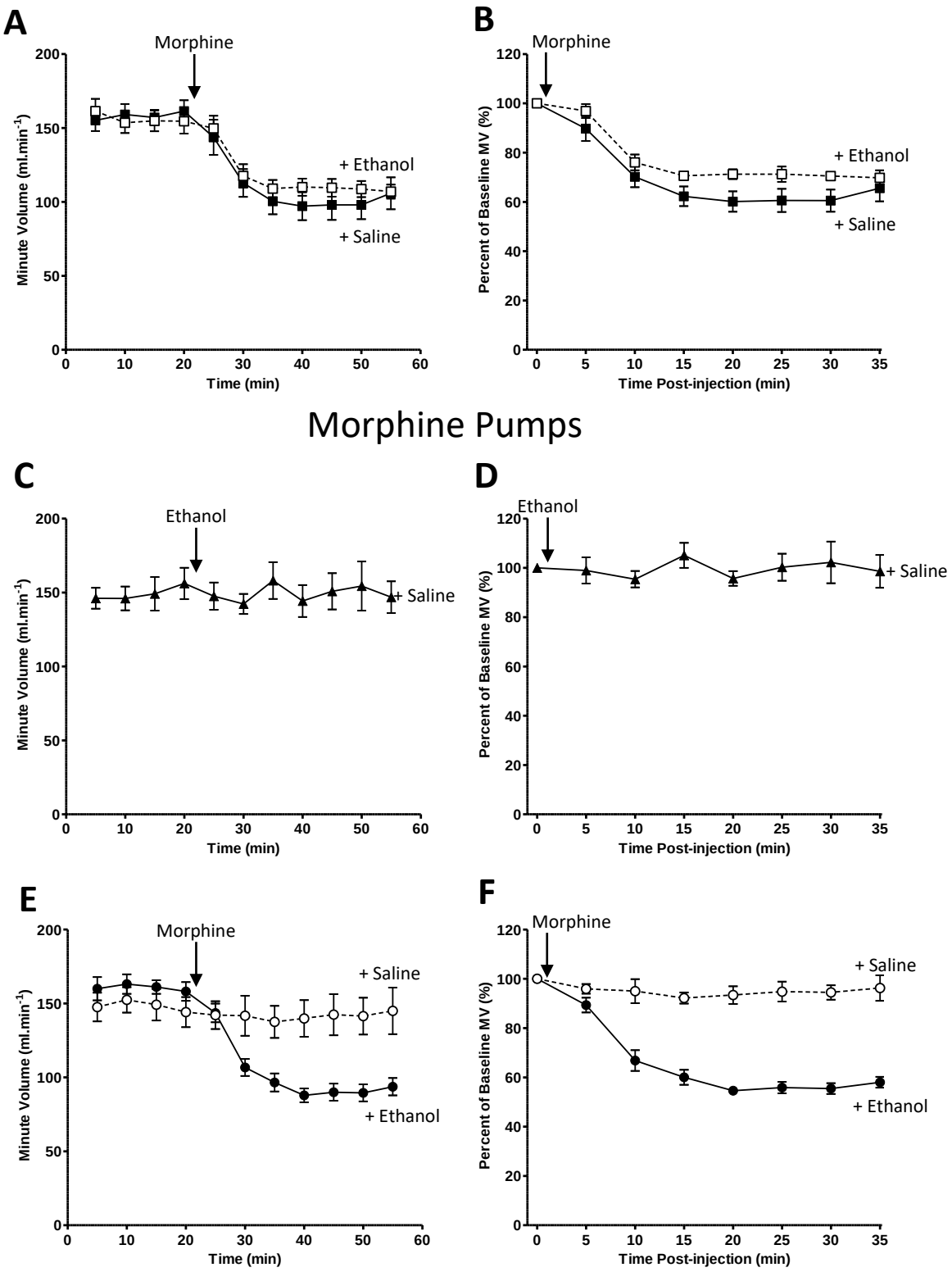


Figure 6.1A-F: Effect of Acute Ethanol on morphine induced tolerance to morphine respiratory depression. *(**A-B**) 6d saline pump implanted mice were injected with ethanol (0.3 g/kg) or saline at the same time as morphine (10 mg/kg). Ethanol did not alter the decrease in MV seen following morphine administration when compared to saline (**G**). (**C-D**) 6d morphine pump implanted mice injected with ethanol and saline at the same time did not cause a change in MV. (**E-F**) Co-administration of morphine and saline in morphine pump implanted mice did not cause a change in MV. Co-administration of ethanol and morphine in morphine pump implanted mice resulted in a significant decrease in MV (**G**) N=7 for all groups.*

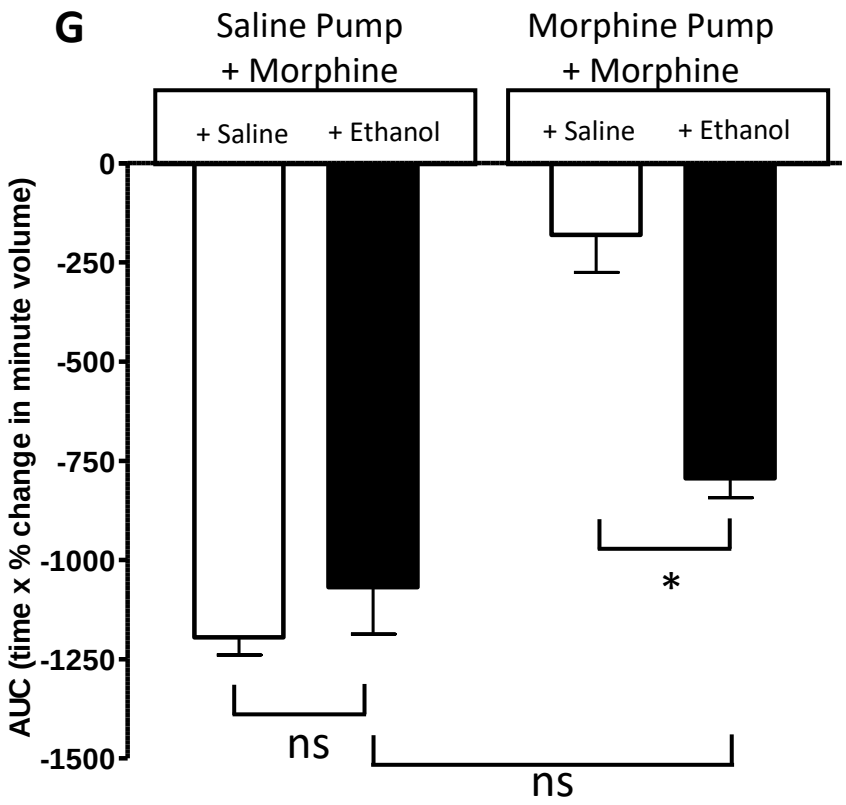


Figure 6.1G: Effect of Acute Ethanol on morphine induced tolerance to morphine respiratory depression*. Ethanol and morphine co-administration in saline pump implanted mice did not significantly alter the decrease in mouse MV compared to saline injected controls. Ethanol and morphine co-administered in morphine pump implanted mice did significantly enhance the decrease in MV induced by morphine. This decrease was not significantly different from saline pump implanted controls. * indicates p<0.05 groups were compared in a two-by-two factorial with Two-way ANOVA followed by Bonferroni's comparison. N=7 for all groups.*

6.2.2 Effect of Acute Ethanol on Medium Oxycodone Induced Tolerance to Morphine Respiratory Depression

Male CD-1 mice received a prolonged treatment of either medium oxycodone or saline through implantation of an osmotic mini-pump for 6 d (See Materials and Methods section 2.4.1). Saline pump-implanted mice were co-administered a morphine challenge (10 mg/kg i.p.) with either saline or ethanol (0.3 g/kg i.p.) (Fig. 6.2A-B Morphine depressed minute volume and the depression of minute volume by morphine was not enhanced or decreased when co-administered with ethanol compared to saline control (Fig. 6.2A-B).

An ethanol challenge, co-administered with saline in medium oxycodone pump-implanted mice did not depress minute volume (Fig. 6.2C-D). Similarly, a morphine challenge, co-administered with saline in medium oxycodone pump-implanted mice did not depress minute volume (Fig. 6.2E-F) and was significantly decreased compared to the effect of morphine administered to saline pump-implanted mice. However, co-administration of a morphine challenge with ethanol in medium oxycodone pump-implanted mice did cause a significant depression of minute volume (Fig. 6.2E-G).

Area under the curve (AUC) values were calculated from figures 6.2B, 6.2D and 6.2F and plotted in figure 6.2G. The depression of minute volume induced by a morphine challenge co-administered with ethanol in morphine pump-implanted mice was not significantly different from the depression of minute volume seen in saline pump implanted mice co-administered morphine and ethanol (Fig. 6.2G).

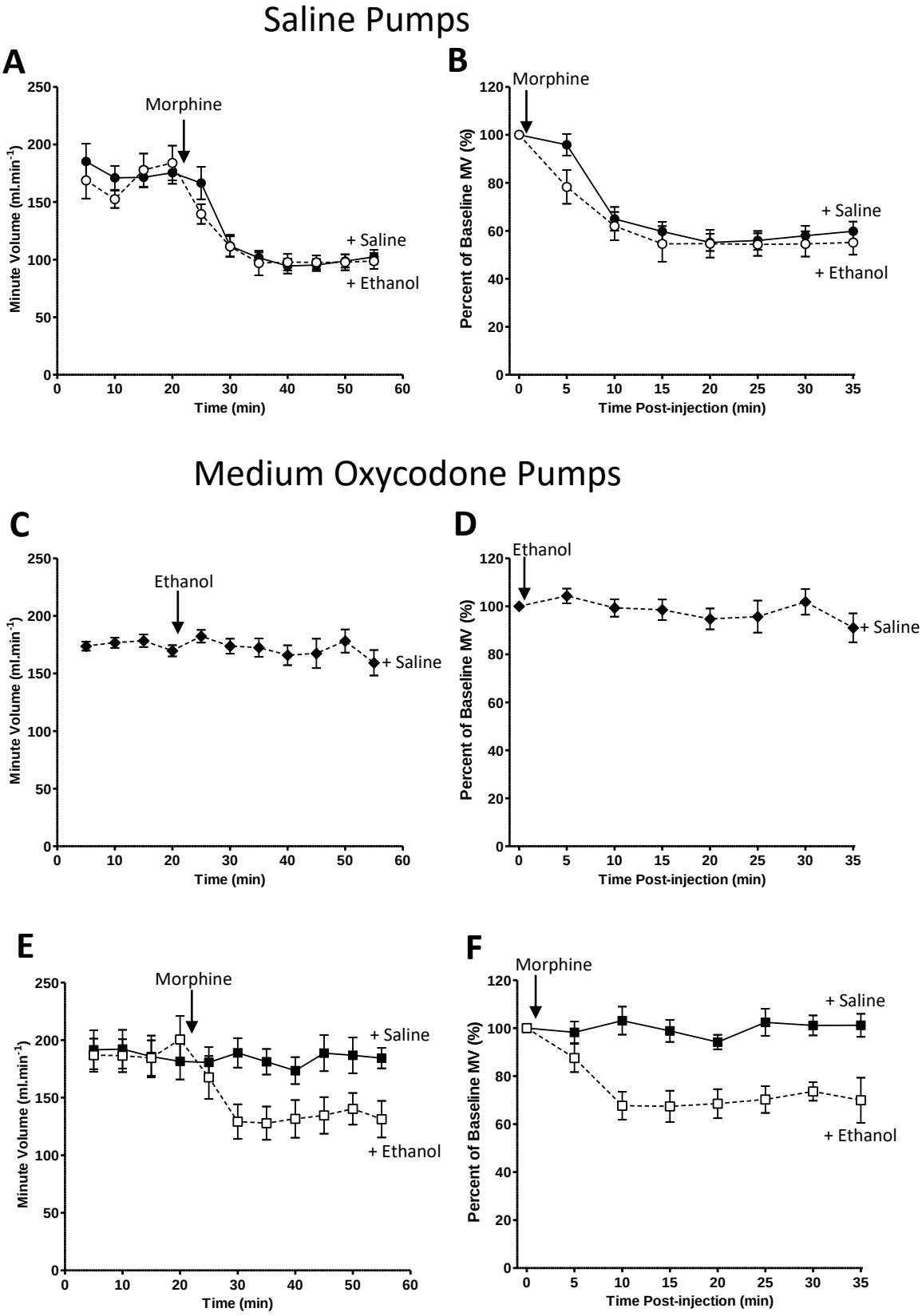


Figure 6.2A-F: Effect of Acute Ethanol on Medium Oxycodone induced tolerance to morphine respiratory depression. *(**A-B**) 6d saline pump implanted mice were injected with ethanol (0.3 g/kg) or saline at the same time as morphine (10 mg/kg). Ethanol did not alter the decrease in MV seen following morphine administration when compared to saline (**G**). (**C-D**) 6d medium oxycodone pump implanted mice injected with ethanol and saline at the same time did not cause a change in MV. (**E-F**) Co-administration of morphine and saline in medium oxycodone pump implanted mice did not cause a change in MV. Co-administration of ethanol and morphine in medium oxycodone pump implanted mice resulted in a significant decrease in MV (**G**) N=7 for all groups.*

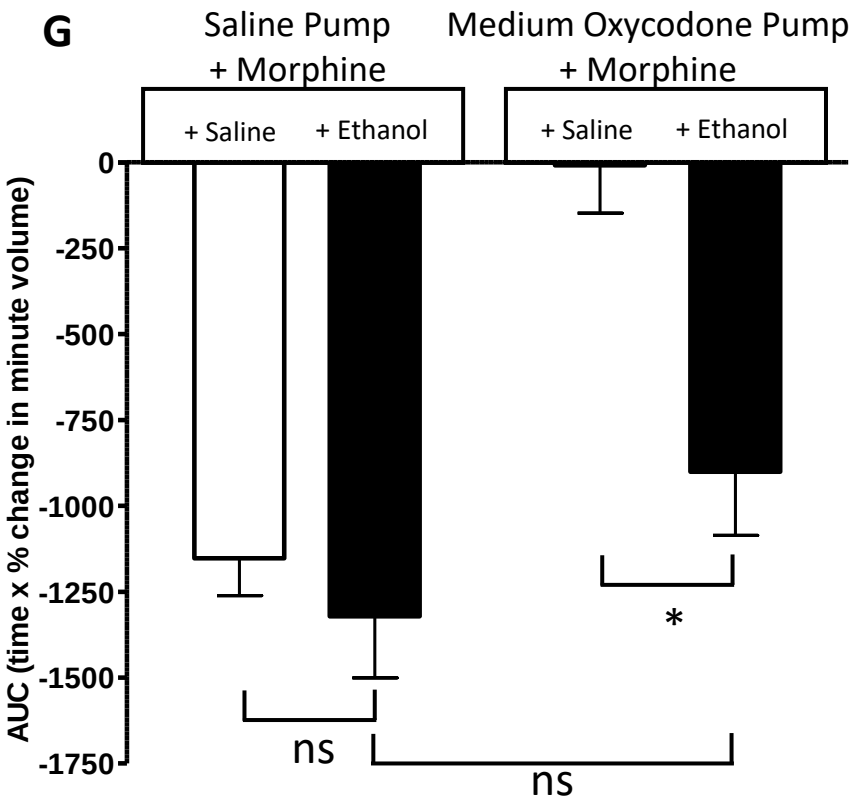


Figure 6.2G: Effect of Acute Ethanol on Medium Oxycodone induced tolerance to morphine respiratory depression. Ethanol and morphine co-administration in saline pump implanted mice did not significantly alter the decrease in mouse MV compared to saline injected controls. Ethanol and morphine co-administered in medium oxycodone pump implanted mice did significantly enhance the decrease in MV induced by morphine. This decrease was not significantly different from saline pump implanted controls. * indicates $p<0.05$ groups were compared in a two-by-two factorial with Two-way ANOVA followed by Bonferroni's comparison. N=7 for all groups.

6.2.3 Effect of Acute Ethanol on High Oxycodone Induced Tolerance to Morphine Respiratory Depression

Male CD-1 mice received a prolonged treatment of either high oxycodone or saline through implantation of an osmotic mini-pump for 6 d (See Materials and Methods section 2.4.1). Morphine (10 mg/kg) challenge co-administered with ethanol (0.3 g/kg) in saline pump-implanted mice depressed minute volume (Fig. 6.3A-B & G). The level of minute volume depression was similar to previous data showing ethanol did not enhance morphine depression of minute volume (Fig. 6.1G & 2G).

An ethanol challenge co-administered with saline in high oxycodone pump-implanted mice did not depress minute volume (Fig. 6.3C-D). Similarly, a morphine challenge co-administered with saline in high oxycodone pump-implanted mice did not depress minute volume (Fig. 6.3E-F) and was significantly decreased compared to the effect of morphine administered to saline pump-implanted mice. When morphine challenge co-administered with ethanol in high oxycodone pump-implanted mice did cause a depression of minute volume (Fig. 6.3E-F), however this was not significant compared to morphine administered alone to high oxycodone pump-implanted mice (Fig. 6.3G).

Previous data has shown that tolerance to morphine respiratory depression induced by high oxycodone pump-implantation in mice is less readily reversed by PKC inhibition than tolerance induced by medium oxycodone pump-implanted mice (See Chapter 5 section 5.3.2). Therefore, as there remains significant tolerance to morphine respiratory depression following high oxycodone pump implantation when the morphine challenge is co-administered with either ethanol or the PKC inhibitor calphostin C, an additional group of high oxycodone pump-implanted mice was administered a morphine challenge co-administered with both ethanol and the PKC inhibitor calphostin C (45 μg/kg).

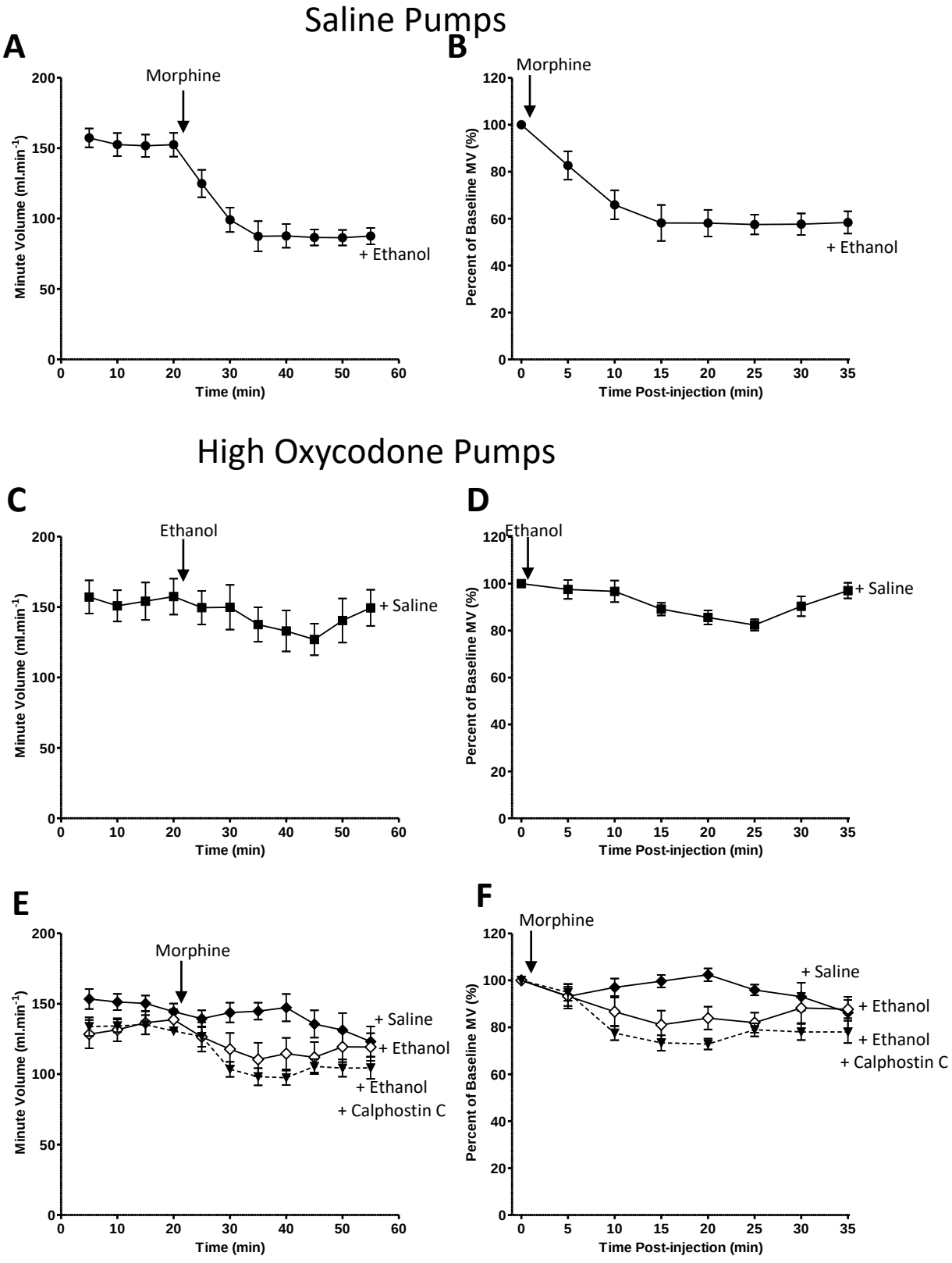


Figure 6.3A-F: Effect of Acute Ethanol on High Oxycodone induced tolerance to morphine respiratory depression. (**A-B**) 6d saline pump implanted mice were injected with ethanol (0.3 g/kg) at the same time as morphine (10 mg/kg). (**C-D**) 6d high oxycodone pump implanted mice injected with ethanol and saline at the same time did not cause a change in MV. (**E-F**) Co-administration of morphine and saline in high oxycodone pump implanted mice did not cause a change in MV. Co-administration of ethanol and morphine in medium oxycodone pump implanted mice did not cause a change in MV. Co-administration of ethanol, calphostin C (45 μg/kg) and morphine in high oxycodone pump mice did cause a significant decrease in mouse MV (**G**) N=7 for all groups.

Morphine, when co-administered with ethanol and calphostin C in high-oxycodone pump-implanted mice, depressed minute volume (Fig. 6.3E-G). The depression of minute volume was significantly greater than morphine co-administered with saline or ethanol co-administered with saline in high-oxycodone pump-implanted mice (Fig. 6.3G). However, the level of minute volume depression was not significantly greater than morphine co-administered with ethanol alone in high oxycodone pump-implanted mice and was still significantly reduced compared to morphine co-administered with ethanol in saline pump-implanted mice (Fig. 6.3G).

These data suggest that at higher induction doses, oxycodone recruits a secondary mechanism of tolerance that is none reversible by ethanol. Given that PKC inhibition was also less effective at reversing high oxycodone induced tolerance to morphine respiratory depression, and that ethanol and PKC inhibition did not act significantly additively, this would suggest that the secondary mechanism is independent of PKC activation.

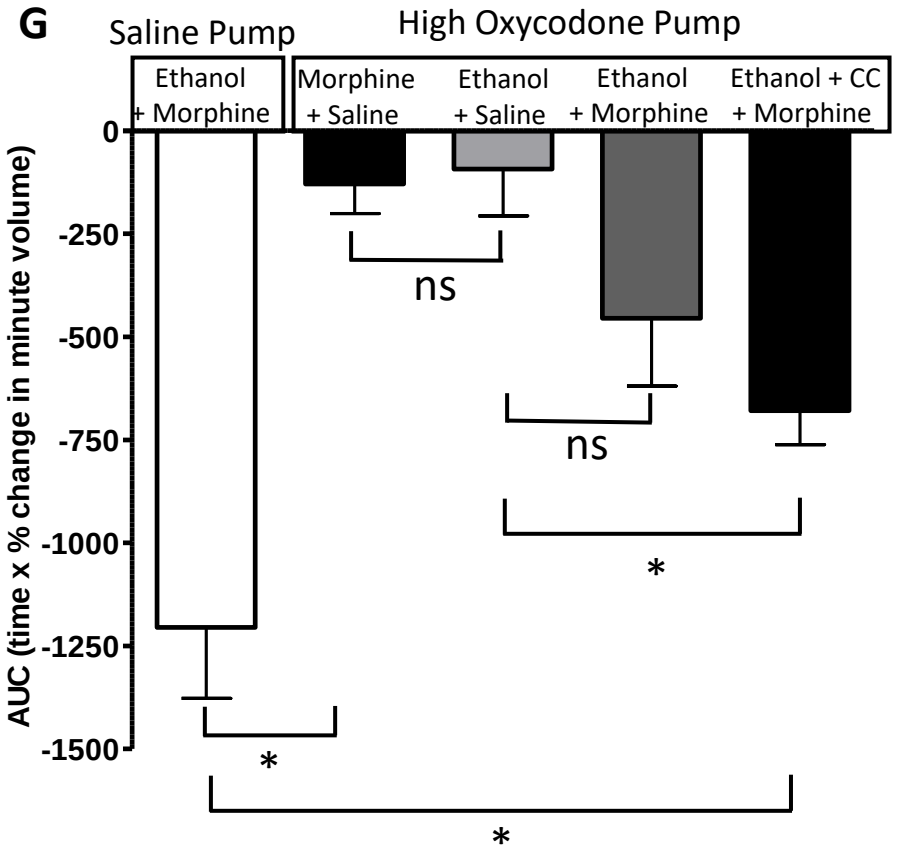


Figure 6.3G: Effect of Acute Ethanol on High Oxycodone induced tolerance to morphine respiratory depression. The decrease in MV induced by morphine and saline co-administered to high oxycodone pump mice was significantly reduced from saline pump control. Ethanol and saline did not decrease MV when co-administered to high oxycodone pump mice. The co-administration of ethanol and morphine did not significantly enhance the decrease in MV compared to morphine administered with saline in high oxycodone pump mice. Co-administration of ethanol, calphostin C and morphine did significantly enhance the decrease in MV seen compared to morphine administered with saline. The decrease in MV was significantly reduced compared to saline pump control. * indicates $p<0.05$ groups were compared with a Two-way ANOVA followed by Bonferroni's comparison. N=7 for all groups.

6.3 Effect of Prolonged Ethanol Diet on Morphine Induced Tolerance to Morphine Respiratory Depression

Polydrug abuse by opioid addicts is a chronic problem, with abuse of multiple illicit drugs as well as alcohol occurring most days. It was therefore considered that an acute injection of ethanol might reflect an extremely artificial system compared to prolonged consumption of ethanol. By extending the period of ethanol consumption in mice we may see little to no effect on tolerance to morphine respiratory depression. This may be due to prolonged ethanol recruiting additional mechanisms as well as desensitization to some effects of ethanol.

In order to investigate the effect of prolonged ethanol on morphine induced tolerance to morphine respiratory depression, a protocol for administering liquid control and ethanol diet was utilized (See Materials and Methods section 2.13.

6.3.1 Consumption of Control and Ethanol Diet

A previous publication has demonstrated that the ethanol diet protocol used in this experiment is palatable to mice and is consumed in equivalent amounts to the control diet fed to mice (Bertola et al., 2013). However, to ensure that mice were feeding equivalent amounts and that mouse weight was maintained, diet consumption was monitored daily, and mouse weight monitored every three days.

As mice were group housed, 4 to a cage, individual diet consumption was not measured, but the average diet consumption (g) per mouse per day was measured. There was no difference in the amount of control and ethanol diet consumed by mice that were implanted with either saline pump or morphine pumps (Fig. 6.4A).

Comparison of group mouse weight on day 0 and on day 16 (the final day) of the experiment demonstrates that overall no weight loss occurred, with no significant change in mouse weight over this period (Fig. 6.4B). No mice were terminated during this experiment for weight loss. Before implantation of saline pumps or morphine pumps in mice fed either control or ethanol diet, baseline respiration was measured for 20 min. Each following day (for 6 days) respiration was measured for 20 min to assess changes in respiration affected by the implantation of saline or morphine pumps.

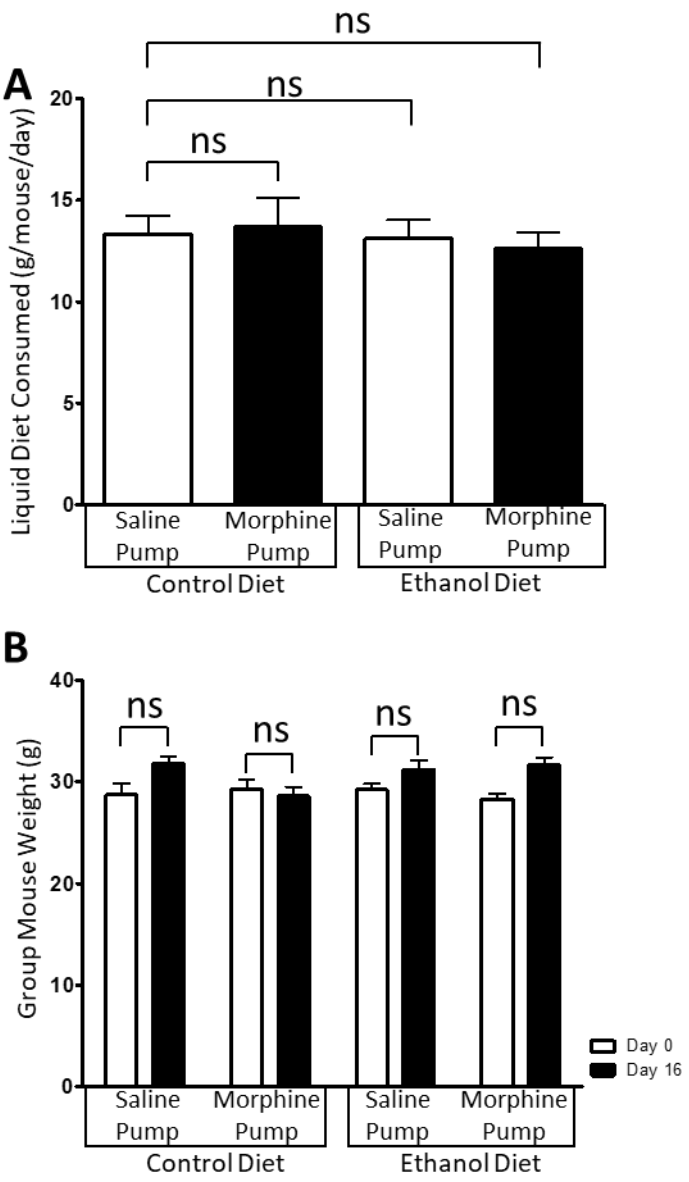


Figure 6.4: Prolonged Ethanol Diet Consumption and Mouse Weight . Mice fed a liquid control diet and implanted with saline pumps did not significantly lose or gain weight over the 16 day experimental protocol (**B**). The consumption of control diet by mice implanted with morphine pumps, and the consumption of ethanol diet by mice implanted with either saline or morphine pumps was not significantly different from control diet-saline pump control mice. No group of mice significantly lost or gained with over the experimental protocol. (**A**) mice were compared by One-way ANOVA with Bonferroni's comparison. (**B**) Beginning and end weights were compared by a two-tailed paired Student's T-test. N=7 for all groups.

6.3.2 Mouse Respiration During Morphine Tolerance Induction

Control diet fed mice implanted with either morphine or saline pumps did not display any significant depression of minute volume following pump implantation compared to pre-implantation baseline (Fig. 6.5A).

Ethanol diet fed mice implanted with a saline pump did not show any significant depression of minute volume following pump implantation when compared to pre-implantation baseline (Fig. 6.5B). However, ethanol diet fed mice implanted with a morphine pump showed a significant reduction in minute volume following implantation compared to both pre-implantation baseline and saline pump implanted mice minute volume (Fig. 6.5B).

On day 6 of pump implantation, baseline minute volume was measured prior to a challenge with morphine. There was no significant difference in the baseline minute volume of control diet fed mice implanted with either saline or morphine pumps, or ethanol diet fed mice implanted with saline pumps on day 6 (Fig. 6.5C). However, ethanol diet fed mice implanted with morphine pumps had a significantly lower baseline minute volume on day 6 compared to control (Fig. 6.5C).

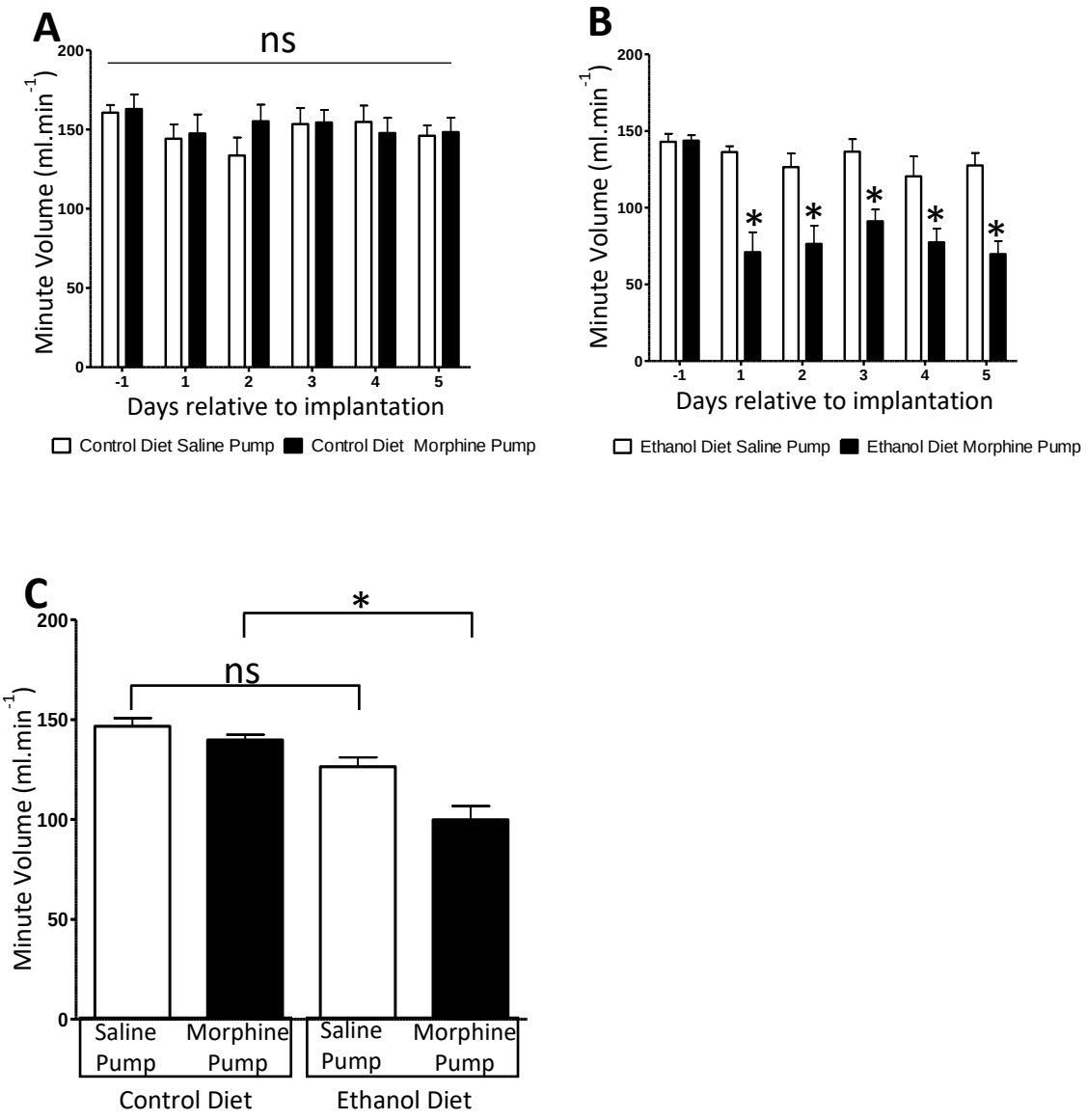


Figure 6.5: Effect of a Prolonged Ethanol Diet on the Development of Morphine Tolerance. (**A**) Implantation of a morphine pump for 6d in control diet fed mice did not cause a significant decrease in mouse MV over the 6d period compared to saline pump implanted mice. (**B**) Implantation of a morphine pump for 6d in ethanol diet fed mice resulted in a significantly decreased MV compared to saline pump implanted mice. (**C**) The baseline respiration of control diet fed mice (saline and morphine pumps) was not significantly different from ethanol diet fed mice implanted with a saline pump on day 6 of implantation. Ethanol diet fed mice implanted with a morphine pump had a significantly lower baseline respiration compared to control diet fed mice implanted with a morphine pump on day 6 of implantation. * indicates $p<0.05$ (**A-B**) Groups compared by Two-way ANOVA with Bonferroni's comparison. (**C**) Groups compared by One-way ANOVA with Bonferroni's comparison. N=7 for all groups.

6.3.3 Effect of Ethanol Diet on the Expression of Tolerance to Morphine Respiratory Depression

Following implantation of saline or morphine pumps for 6 d, all mice were administered an acute challenge dose of morphine (10 mg/kg). The acute morphine challenge, administered to saline pump implanted mice, fed either a control or ethanol diet significantly depressed mouse minute volume (Fig. 6.6A-B). The degree of minute volume depression by the morphine challenge was not significantly different between ethanol or control diet fed mice (Fig. 6.6E).

The acute morphine challenge, administered to control diet fed, morphine pump implanted mice did not depress minute volume (Fig. 6.6C-D). The effect of acute morphine on mouse minute volume in control diet fed, morphine pump implanted mice was significantly reduced compared to the depression of minute volume in control diet fed, saline pump implanted mice (Fig. 6.6E).

However, the acute morphine challenge produced a significant depression of minute volume in ethanol diet fed mice, implanted with a morphine pump (Fig. 6.6C-E). The degree of acute morphine depression of minute volume was not significantly different between ethanol diet fed mice implanted with either saline or morphine pumps (Fig. 6.6E).

These data collectively suggest that a prolonged ethanol diet prevented the development of tolerance to morphine respiratory depression, and revealed the depression of minute volume caused by morphine released from the implanted morphine pump. Additionally, ethanol diet fed mice implanted with a morphine pump remained sensitive to an additional on top dose of acute morphine. This suggests that prolonged consumption of ethanol by heroin addicts is likely to have profound consequences on the development of tolerance to the respiratory depressant effects of heroin.

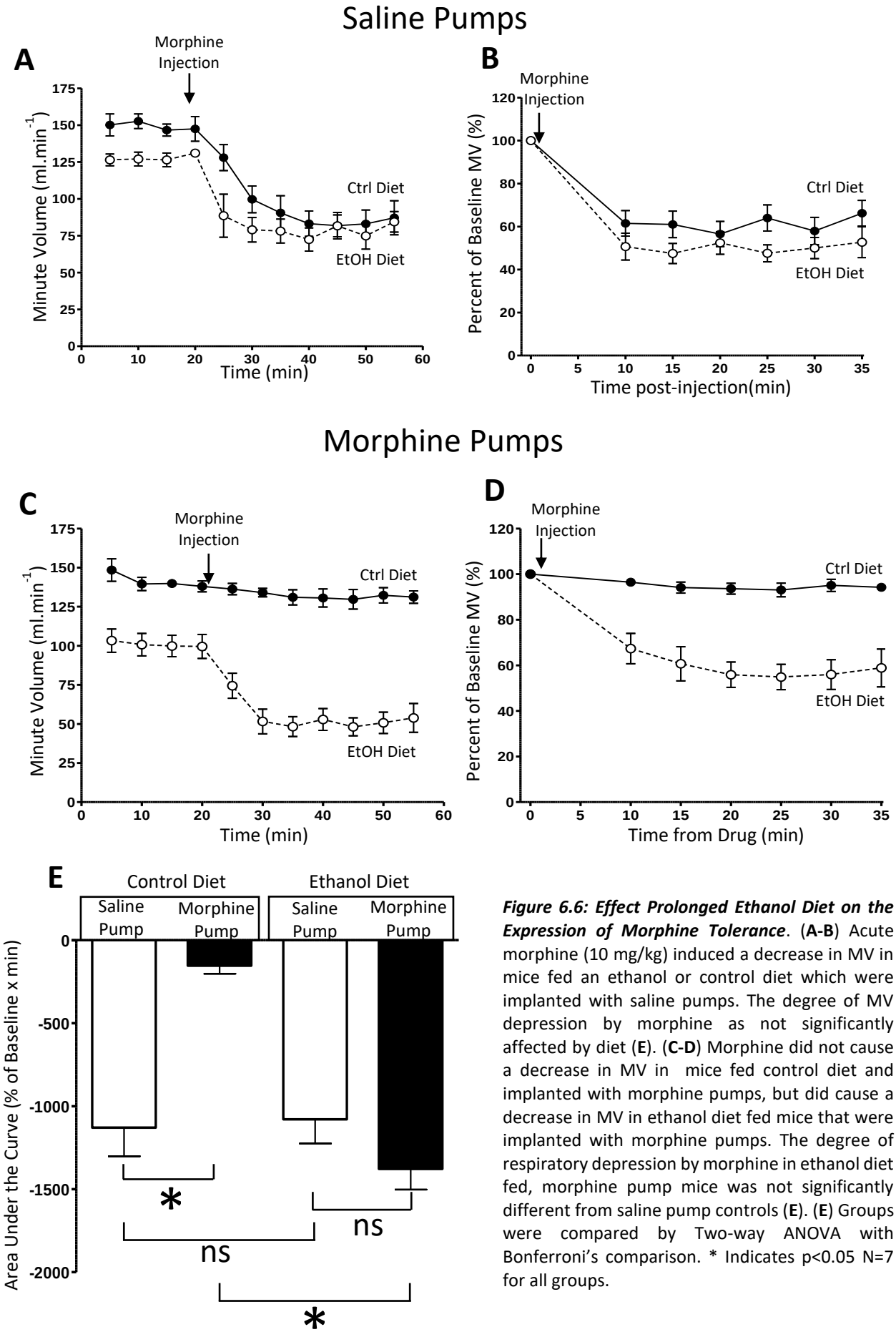


Figure 6.6: Effect Prolonged Ethanol Diet on the Expression of Morphine Tolerance. (**A-B**) Acute morphine (10 mg/kg) induced a decrease in MV in mice fed an ethanol or control diet which were implanted with saline pumps. The degree of MV depression by morphine as not significantly affected by diet (**E**). (**C-D**) Morphine did not cause a decrease in MV in mice fed control diet and implanted with morphine pumps, but did cause a decrease in MV in ethanol diet fed mice that were implanted with morphine pumps. The degree of respiratory depression by morphine in ethanol diet fed, morphine pump mice was not significantly different from saline pump controls (**E**). (**E**) Groups were compared by Two-way ANOVA with Bonferroni's comparison. * Indicates $p<0.05$ N=7 for all groups.

6.3.4 Effect of Ethanol Diet on Morphine Distribution

Prolonged ethanol administration in mice often results in damage to first pass metabolic pathways within the periphery, such as the kidney and liver, however the length of ethanol diet administration chosen for this experiment was not thought to result in significant damage to this system (Bertola et al., 2013), but it is still possible that the consumption of this ethanol diet might have resulted in altered morphine metabolism to account for the changes seen in acute respiratory responses to morphine.

Additionally, the implantation of a morphine pump may alter the overall metabolic rate of ethanol consumed from an ethanol diet, again providing a potential reason for the behavioural outcomes seen. To investigate these potentially confounding factors, blood samples were analysed for both ethanol and morphine content and brain samples were analysed for morphine content (See Materials and Methods section 2.14 & 2.15). An analysis of ethanol brain levels was not possible.

Plasma morphine levels were not significantly different in control or ethanol diet fed mice implanted with morphine pumps (Fig. 6.7A). Similarly, brain morphine levels were not significantly different in control or ethanol diet fed mice implanted with morphine pumps (Fig. 6.7B). These data indicate that this ethanol diet protocol did not alter the base metabolism or distribution of morphine in mice implanted with a morphine pump for 6 d.

Plasma ethanol levels were not significantly different in ethanol diet fed mice implanted with saline or morphine pumps (Fig. 6.7C). This indicates that the morphine pump protocol used did not alter the plasma ethanol content of mice and is therefore unlikely to have altered ethanol metabolism or ethanol distribution.

All blood and brain samples were taken on the final day of the experiment following measurement of respiratory depression to acute morphine. Therefore, these data only substantiate that 14 days following ethanol diet consumption that there were not differences in morphine concentrations in brain or plasma. However, it is possible that sampling of blood and brain taken earlier on in diet administration or pump implantation may reveal differences.

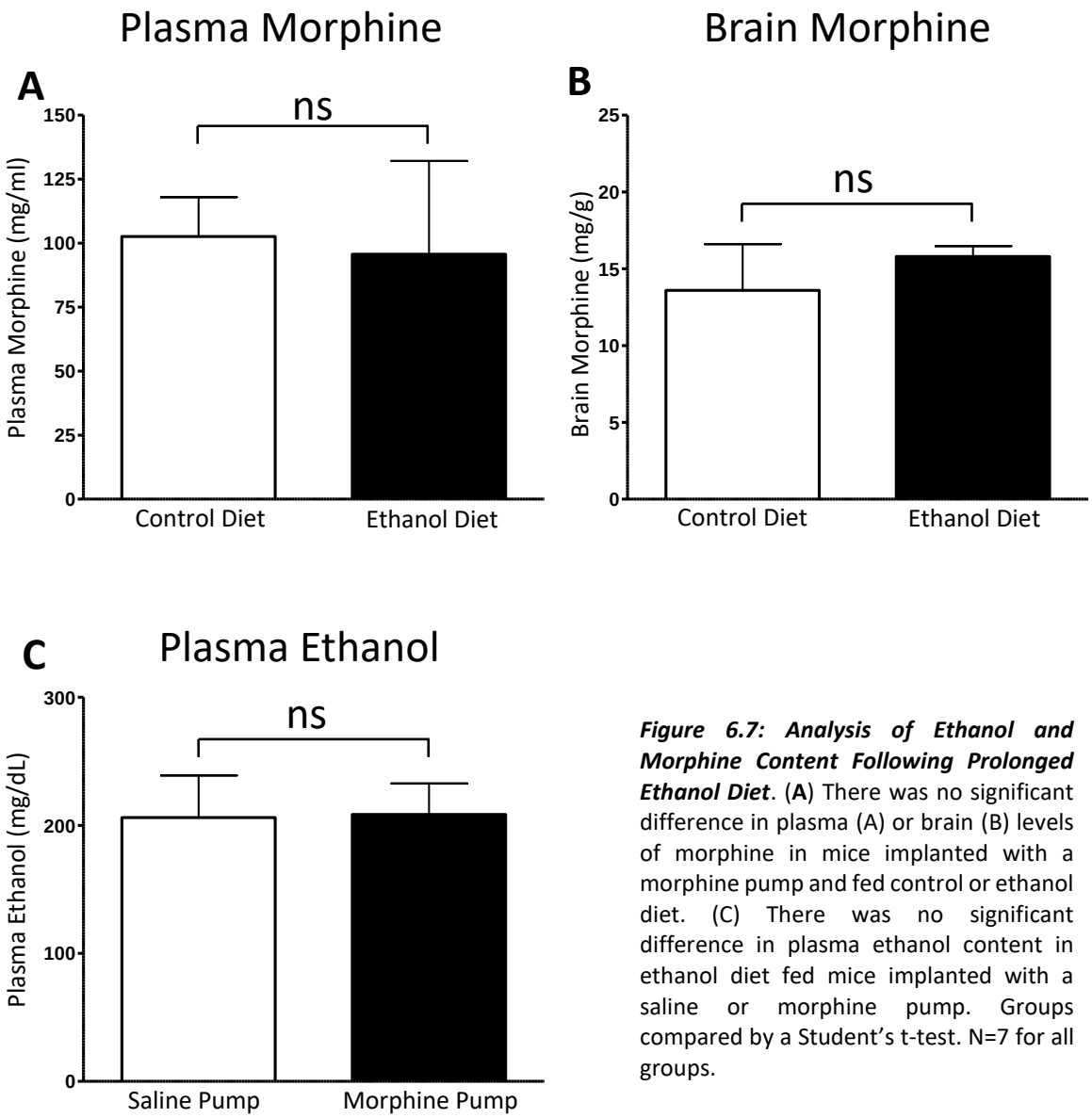


Figure 6.7: Analysis of Ethanol and Morphine Content Following Prolonged Ethanol Diet. (**A**) There was no significant difference in plasma (A) or brain (B) levels of morphine in mice implanted with a morphine pump and fed control or ethanol diet. (C) There was no significant difference in plasma ethanol content in ethanol diet fed mice implanted with a saline or morphine pump. Groups compared by a Student's t-test. N=7 for all groups.

6.4 Effect of Acute Acetaldehyde on Morphine Induced Tolerance to Morphine Respiratory Depression

Previous data from this authors Masters (by Research) thesis demonstrated that pre-treatment with the acetaldehyde chelator, D-penicillamine, significantly reduced ethanol reversal of tolerance to morphine respiratory depression. That data has been reproduced in this thesis for context (Fig. 6.8A-B). In this experiment, ethanol (0.3 g/kg) was co-administered with a morphine challenge (10 mg/kg) in mice tolerant to morphine with either a saline or D-penicillamine (50 mg/kg) treatment 30 min prior to the morphine/ethanol challenge. Ethanol and morphine produced a significantly greater level of respiratory depression than morphine alone in morphine tolerant mice (Fig. 6.8A-B), but this was significantly attenuated with D-penicillamine treatment (Fig. 6.8A-B).

To further investigate acetaldehyde as a potential mediator of ethanol reversal of morphine tolerance, acetaldehyde was co-administered acutely with morphine in morphine tolerant mice.

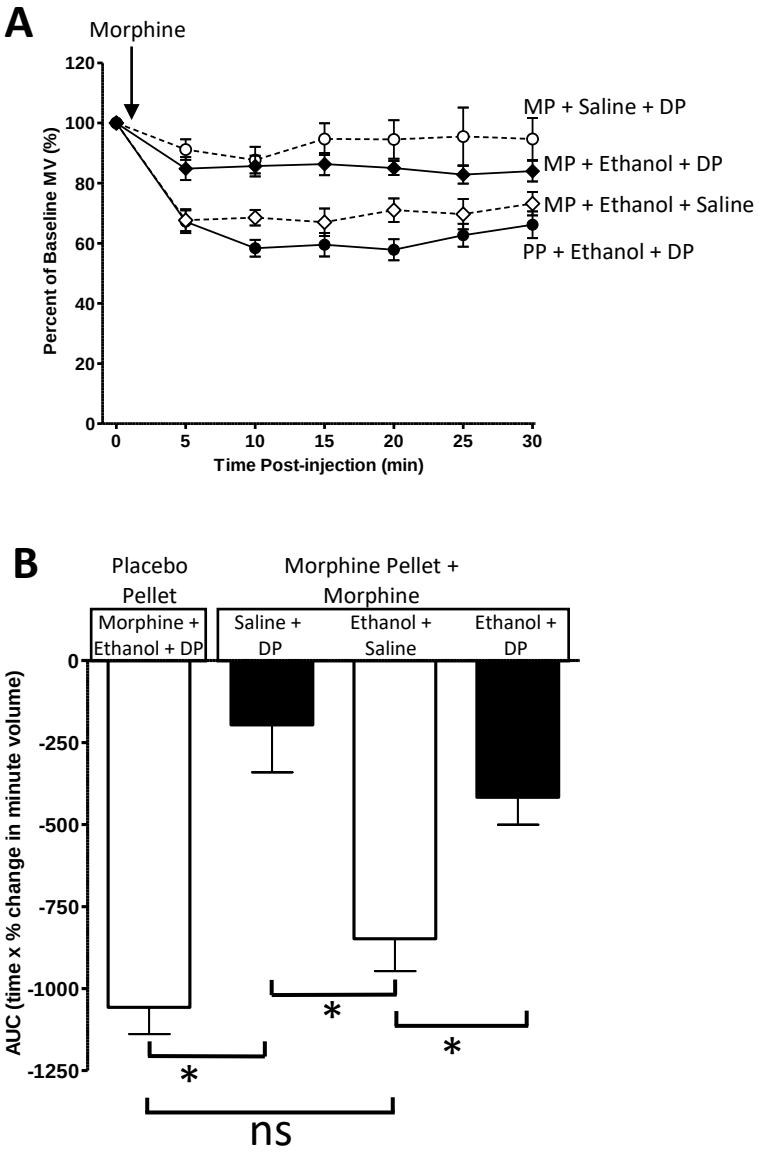


Figure 6.8: Effect of D-Penicllamine on Acute Ethanol Reversal of Morphine induced tolerance to morphine respiratory depression. (**A**) 6d morphine pellet treatment (MP) reduced morphine induced decrease in MV compare to placebo pellet control (PP). Co-administration of ethanol and morphine produced a large decrease in MV. (**B**) MP treatment significantly reduced morphine induced decrease in MV. Ethanol co-administration with morphine produced a significant decrease in MV that was not significant from PP controls. Pre-treatment with D-penicillamine (DP 50 mg/kg for 30 min) significantly attenuated ethanol reversal of morphine tolerance. * indicates $p<0.05$ groups were compared by One-way ANOVA followed by Bonferroni's comparison. N=6 for all groups.

6.4.1 Morphine Pellet Induced Tolerance

Male CD-1 mice were implanted with either a placebo or morphine (75 mg) pellet for 6 d in these experiments (See Materials and Methods section 2.4.1). Tolerance was induced by implantation of 75 mg morphine pellets in these experiments to allow comparison with previously generated data that used this tolerance induction protocol. All acute morphine challenge doses in this experiment were at administered at 10 mg/kg.

An acute challenge dose of morphine administered to placebo pellet-implanted mice, depressed minute volume over the 30-min post morphine period (Fig. 6.9A-B). In comparison, the same dose of morphine did not depress minute volume in morphine pellet-implanted mice (Fig. 6.9A-B) due the development of tolerance. The reduction in minute volume depression was significant (Fig. 6.9G).

6.4.2 Effect of Acetaldehyde on Morphine Pellet Induced Tolerance

Co-administration of acetaldehyde (50 mg/kg) (acetaldehyde-50) and saline or acetaldehyde (100 mg/kg) (acetaldehyde-100) and saline in morphine pellet-implanted mice did not depress minute volume (Fig. 6.9C-D). However, acetaldehyde-50 co-administered with an acute morphine challenge, in morphine pellet-implanted mice, did depress minute volume (Fig. 6.9E-F). Acetaldehyde-50 and morphine co-administered in morphine pellet-implanted mice depressed minute volume to a significantly greater degree than acetaldehyde-50 or morphine alone (Fig. 6.9G). The degree of minute volume depression was, however, still significantly reduced compared to morphine administered to placebo pellet-implanted mice (Fig. 6.9G).

Acetaldehyde-100 co-administered with morphine in morphine pellet-implanted mice, also depressed minute volume (Fig. 6.9E-F). Acetaldehyde-100 and morphine co-administered in morphine pellet-implanted mice depressed minute volume to a significantly greater degree than acetaldehyde-100 or morphine alone (Fig. 6.9G). Additionally, acetaldehyde-100 co-administered with morphine depressed the minute volume of morphine pellet-implanted mice to a significantly greater degree than acetaldehyde-50 co-administered with morphine (Fig. 6.9G). Acetaldehyde-100 and morphine depressed the minute volume of morphine pellet-implanted mice to the same degree as morphine alone depressed minute volume in placebo-pellet implanted mice.

These data demonstrate that acetaldehyde is able to reverse morphine induced tolerance to morphine respiratory depression in the absence of its precursor molecule ethanol. These data indicate that acetaldehyde is likely to play a significant role in ethanol reversal of opioid tolerance.

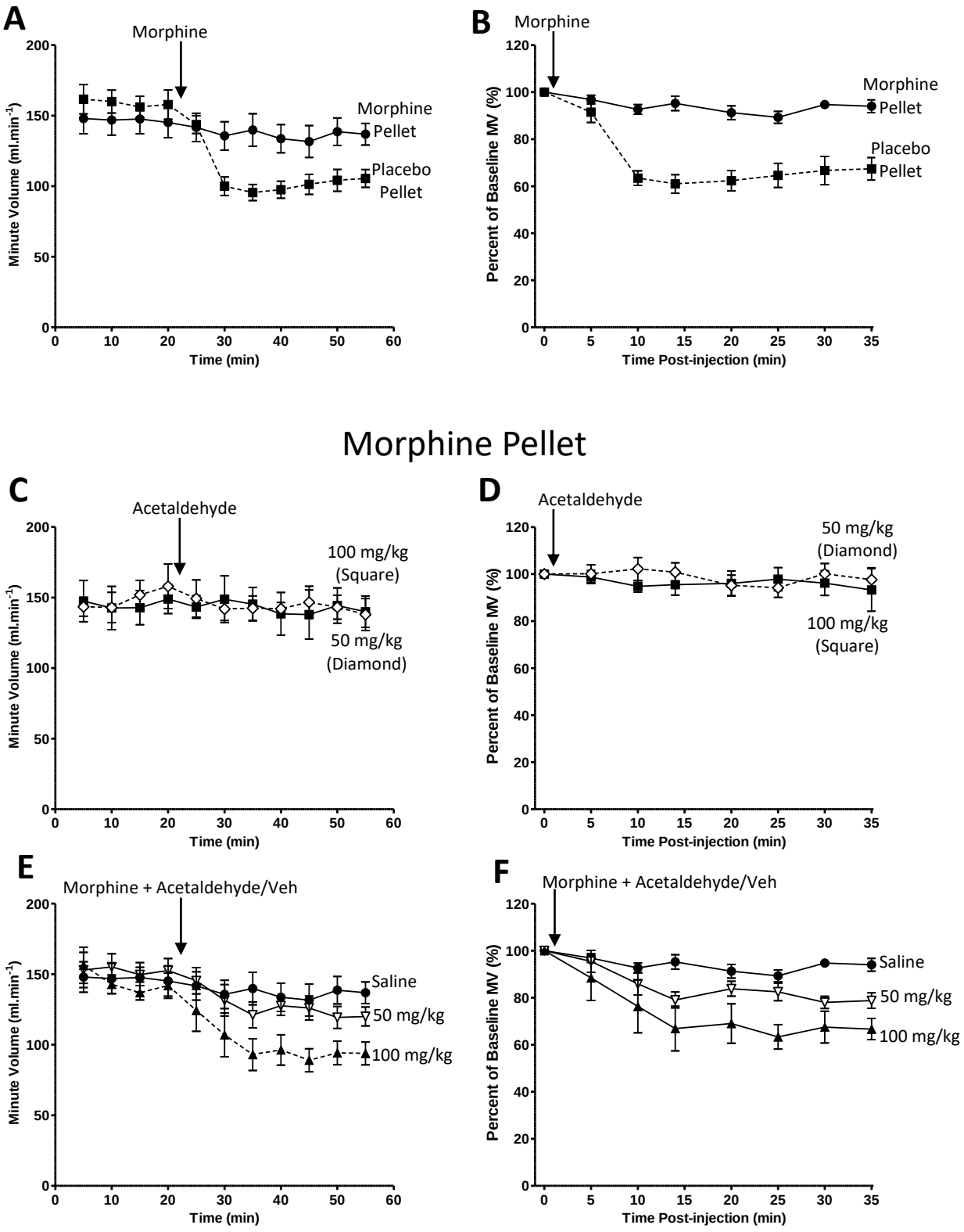


Figure 6.9A-F: Effect of Acute Acetaldehyde on Morphine induced tolerance to morphine respiratory depression. (**A-B**) Morphine (10 mg/kg) did not cause a decrease in mouse MV when administered to mice implanted with a 75 mg morphine pellet for 6d, compared to a marked decrease in MV in mice implanted with a placebo pellet for 6 days. (**C-D**) Acetaldehyde (50 & 100 mg/kg) did not cause a decrease in MV when administered to mice implanted with a morphine pellet for 6d. Acetaldehyde dose-dependently revealed a morphine decrease in MV when co-administered with morphine. N=6 for all groups.

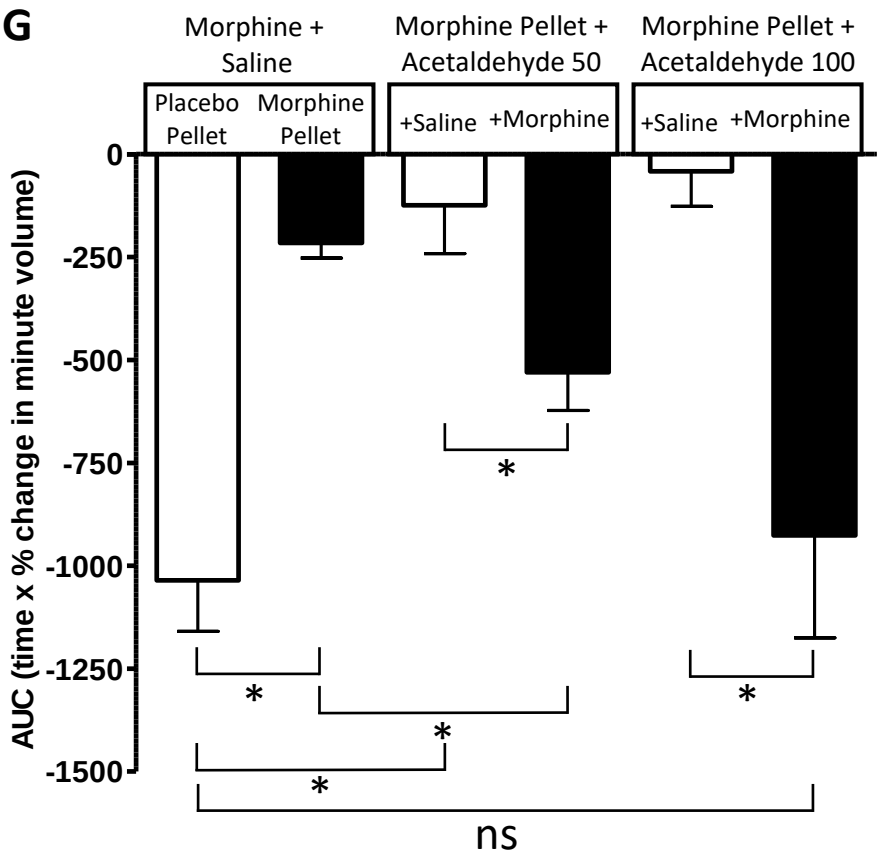


Figure 6.9G: Effect of Acute Acetaldehyde on Morphine induced tolerance to morphine respiratory depression. 6d morphine pellet treatment significantly reduced morphine induced decrease in MV. Morphine induced a significant decrease in MV when co-administered with acetaldehyde (50 mg/kg) compared to saline controls. This was still significantly reduced compared to placebo pellet controls. Morphine co-administered with acetaldehyde (100 mg/kg) induced significant decrease in MV that was not significantly different from placebo pellet controls. * indicates $p<0.05$ groups were compared in a two-by-two factorial with Two-way ANOVA followed by Bonferroni's comparison. N=6 for all groups.

6.5 Acute Pregabalin

6.5.1 Effect of Acute Pregabalin on Morphine Induced Tolerance to Morphine Respiratory Depression

Male CD-1 mice received a prolonged treatment of either morphine or saline through implantation of an osmotic mini-pump for 6 d (See Materials and Methods section 2.4.1). Saline pump-implanted mice were co-administered a morphine challenge (10 mg/kg i.p.) with either saline or pregabalin (20 mg/kg i.p.) (Fig. 6.10A-B). Morphine depressed minute volume and the depression of minute volume by morphine was not enhanced or decreased when co-administered with pregabalin compared to saline control (Fig. 6.10A-B). The baseline minute volume of the morphine and pregabalin administered group was also not significantly different from the baseline minute volume of the morphine and saline administered group.

A pregabalin challenge, co-administered with saline in morphine pump-implanted mice did not depress minute volume (Fig. 6.10C-D). Similarly, a morphine challenge, co-administered with saline in morphine pump-implanted mice did not depress minute volume (Fig. 6.10E-F) and was significantly decreased compared to the effect of morphine administered to saline pump-implanted mice. However, co-administration of a morphine challenge with pregabalin in morphine pump-implanted mice did cause a significant depression of minute volume (Fig. 6.10E-G).

Area under the curve (AUC) values were calculated from figures 6.10B, 6.10D and 6.10F and plotted in figure 6.10G. The depression of minute volume induced by a morphine challenge co-administered with pregabalin in morphine pump-implanted mice was not significantly different from the depression of minute volume seen in saline pump implanted mice co-administered morphine and pregabalin (Fig. 6.10G).

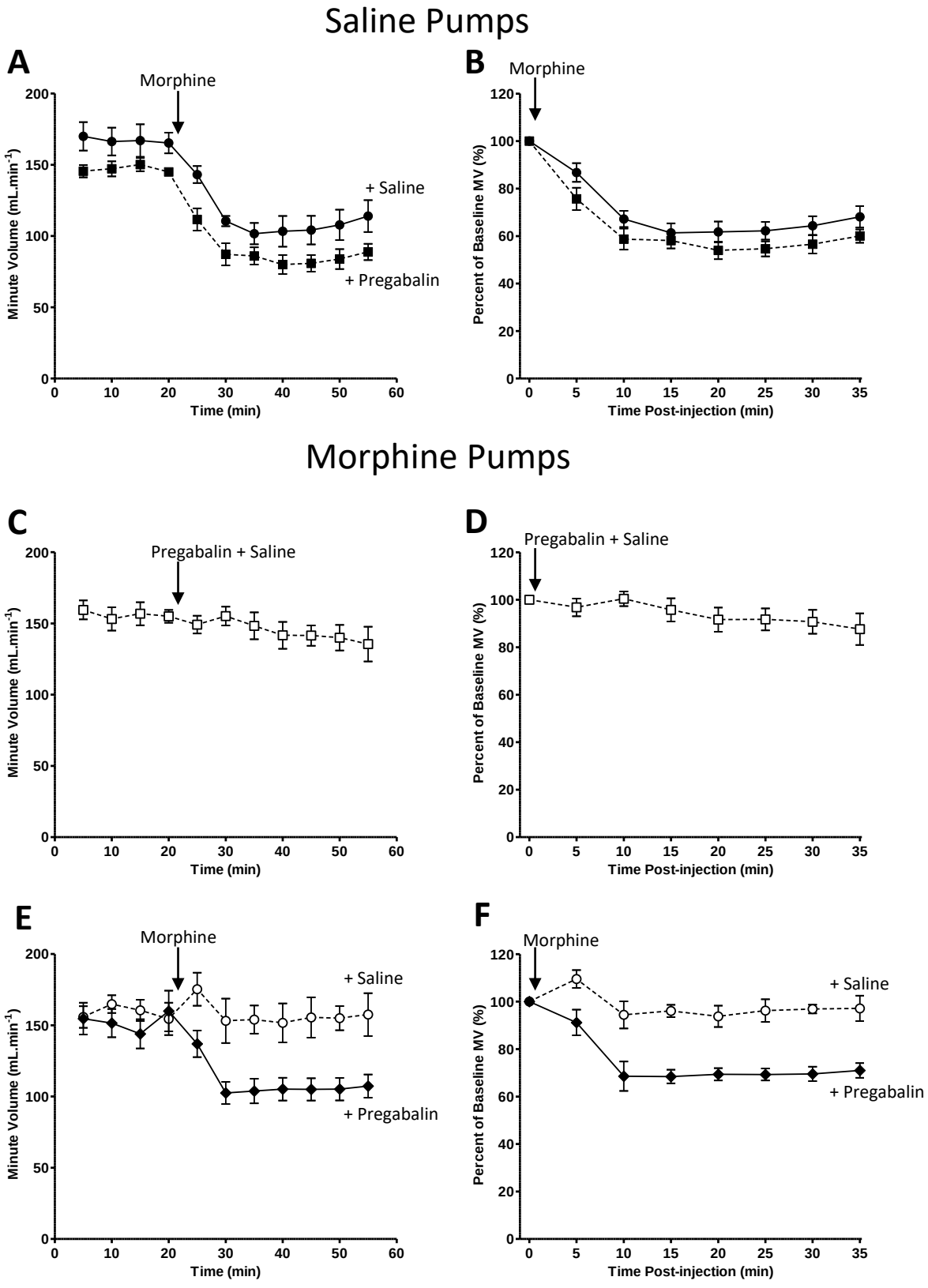


Figure 6.10A-F: Effect of Acute Pregabalin on morphine induced tolerance to morphine respiratory depression. (**A-B**) 6d saline pump implanted mice were injected with pregabalin (20 mg/kg) or saline at the same time as morphine (10 mg/kg). Pregabalin did not alter the decrease in MV seen following morphine administration when compared to saline (**G**). (**C-D**) 6d morphine pump implanted mice injected with pregabalin and saline at the same time did not cause a change in MV. (**E-F**) Co-administration of morphine and saline in morphine pump implanted mice did not cause a change in MV. Co-administration of pregabalin and morphine in morphine pump implanted mice resulted in a significant decrease in MV (**G**) N=7 for all groups.

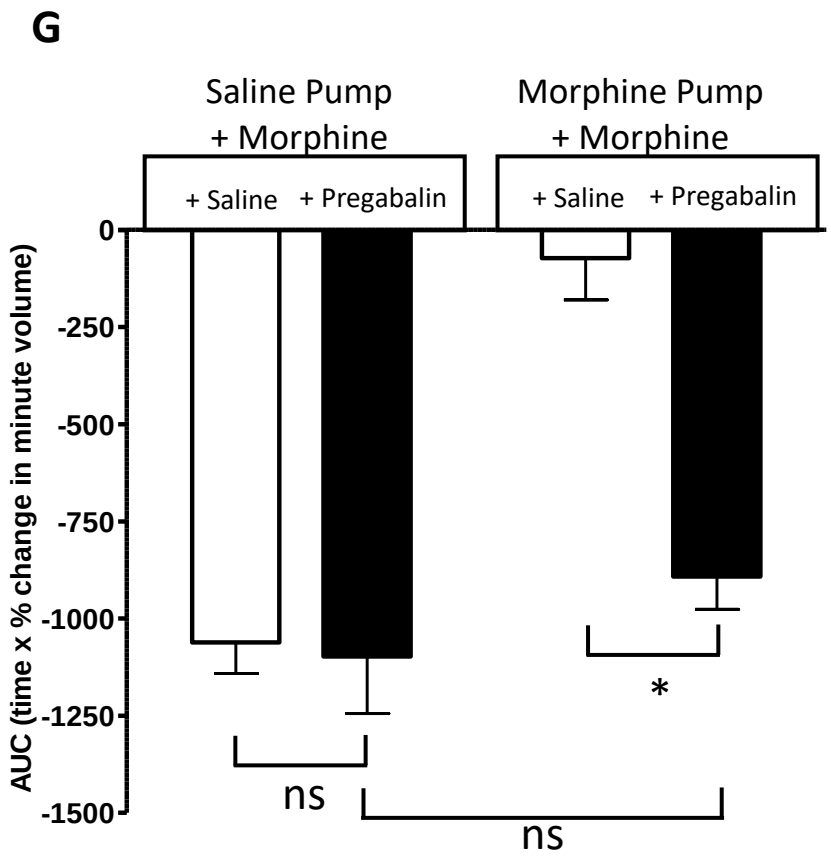


Figure 6.10G: Effect of Acute Pregabalin on morphine induced tolerance to morphine respiratory depression. Pregabalin and morphine co-administration in saline pump implanted mice did not significantly alter the decrease in mouse MV compared to saline injected controls. Pregabalin and morphine co-administered in morphine pump implanted mice did significantly enhance the decrease in MV induced by morphine. This decrease was not significantly different from saline pump implanted controls. * indicates $p<0.05$ groups were compared in a two-by-two factorial with Two-way ANOVA followed by Bonferroni's comparison. N=7 for all groups.

6.5.2 Effect of Acute Pregabalin on Medium Oxycodone Induced Tolerance to Morphine Respiratory Depression

Male CD-1 mice received a prolonged treatment of either medium oxycodone or saline through implantation of an osmotic mini-pump for 6 d (See Materials and Methods section 2.4.1). Saline pump-implanted mice were co-administered a morphine challenge (10 mg/kg i.p.) with saline (Fig. 6.11A-B). Morphine depressed minute volume. Morphine and pregabalin (20 mg/kg i.p.) was not administered to saline pump-implanted mice as this had pregabalin had already been demonstrated to not enhance morphine depression of minute volume in a parallel experiment (See Fig. 6.10A-B).

A pregabalin challenge, co-administered with saline in medium oxycodone pump-implanted mice did not depress minute volume (Fig. 6.11C-D). Similarly, a morphine challenge, co-administered with saline in medium oxycodone pump-implanted mice did not depress minute volume (Fig. 6.11C-D) and was significantly decreased compared to the effect of morphine administered to saline pump-implanted mice. However, co-administration of a morphine challenge with pregabalin in medium oxycodone pump-implanted mice did cause a significant depression of minute volume (Fig. 6.11C-D).

Area under the curve (AUC) values were calculated from figures 6.11B and 6.11D and plotted in figure 6.11E. The depression of minute volume induced by a morphine challenge co-administered with pregabalin in morphine pump-implanted mice was not significantly different from the depression of minute volume seen in saline pump implanted mice co-administered morphine and saline (Fig. 6.11E).

Co-administration of morphine (10 m/kg) and saline to saline pump implanted mice resulted in a large decrease in mouse MV (Fig. 6.11A-B).

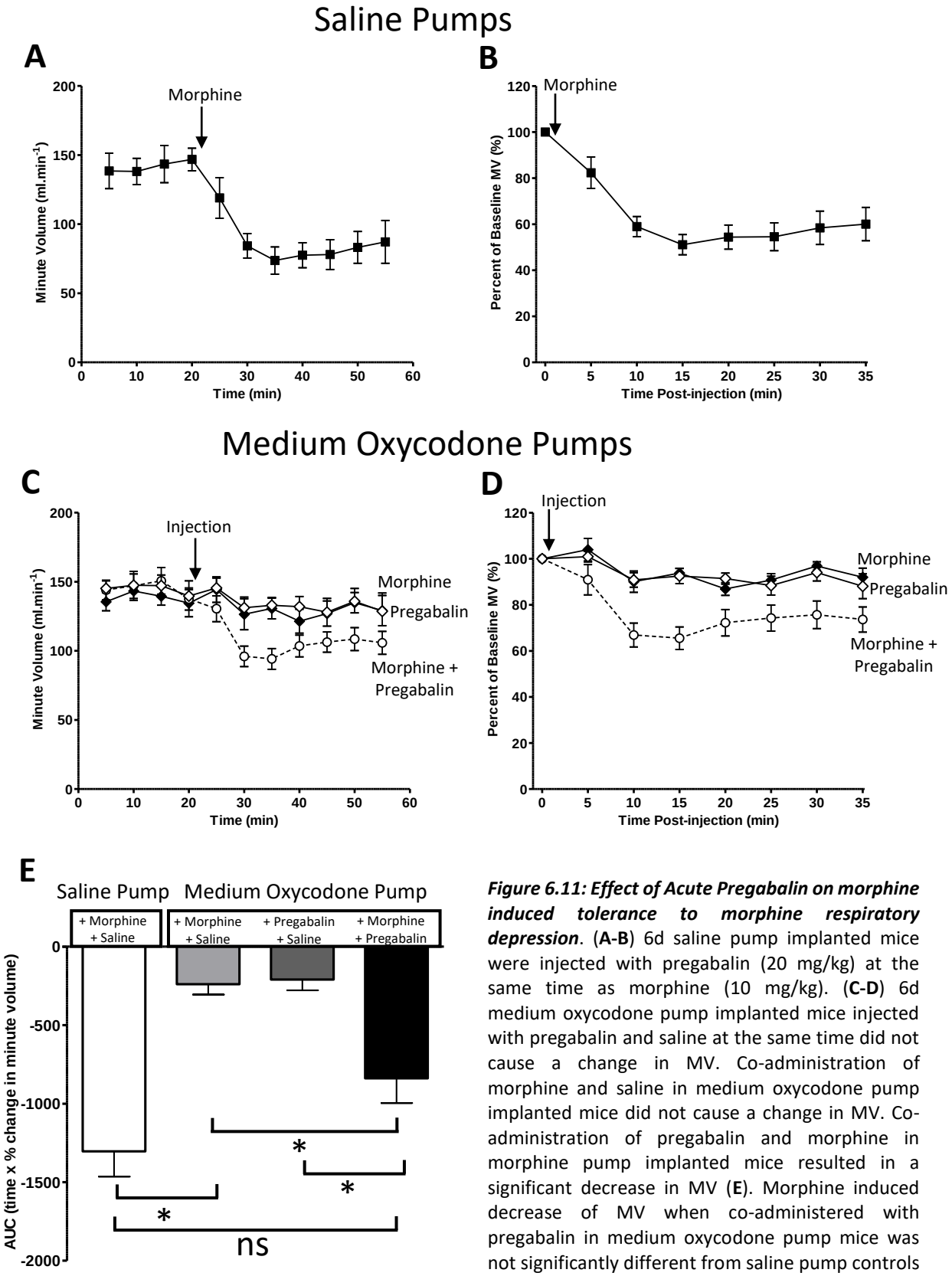


Figure 6.11: Effect of Acute Pregabalin on morphine induced tolerance to morphine respiratory depression. (**A-B**) 6d saline pump implanted mice were injected with pregabalin (20 mg/kg) at the same time as morphine (10 mg/kg). (**C-D**) 6d medium oxycodone pump implanted mice injected with pregabalin and saline at the same time did not cause a change in MV. Co-administration of morphine and saline in medium oxycodone pump implanted mice did not cause a change in MV. Co-administration of pregabalin and morphine in morphine pump implanted mice resulted in a significant decrease in MV (**E**). Morphine induced decrease of MV when co-administered with pregabalin in medium oxycodone pump mice was not significantly different from saline pump controls (**E**). Groups were compared by One-way ANOVA with Bonferroni's comparison. * Indicates $p<0.05$. N=7 for all groups.

6.5.3 Effect of Acute Pregabalin on High Oxycodone Induced Tolerance to Morphine Respiratory Depression

Male CD-1 mice received a prolonged treatment of either high oxycodone or saline through implantation of an osmotic mini-pump for 6 d (See Materials and Methods section 2.4.1). Co-administration of morphine (10 m/kg) and saline to saline pump implanted mice resulted in a depression of minute volume (Fig. 6.12A-B). Co-administration of morphine with both ethanol (0.3 g/kg) and pregabalin (20 mg/kg) to saline pump implanted mice also produced a depression of minute volume, however this was not significantly greater than the depression of minute volume induced by co-administration of morphine and saline (Fig. 6.12G).

Morphine, co-administered with two injections of saline in high oxycodone pump-implanted mice did not depress minute volume (Fig. 6.12C-D). Similarly, pregabalin co-administered with two injections of saline in high oxycodone pump-implanted mice did not depress minute volume (Fig. 6.12C-D). The co-administration of morphine with pregabalin and saline also did not depress minute volume (Fig. 6.12C-D), with no significant increase in minute volume depression by morphine co-administered with pregabalin compared to either administered separately (Fig. 6.12G).

Both ethanol (See Fig. 6.3G) and pregabalin have a relatively diminished ability to reverse high oxycodone induced tolerance to morphine respiratory depression when compared to tolerance to morphine induced by medium oxycodone pumps which is reversible by both ethanol (see Fig. 6.2G) and pregabalin (See Fig. 6.11E). As ethanol is generally freely available and pregabalin is not currently a strictly regulated drug, it is likely that co-consumption of ethanol and pregabalin may occur. Therefore, it was hypothesized that co-administration of ethanol, pregabalin and morphine may be capable of reversing tolerance induced by high oxycodone pump administration.

Indeed, co-administration of ethanol, pregabalin and morphine to mice implanted with high oxycodone pumps for 6d did decrease depress minute volume (Fig. 6.12E-F) and this was significantly greater than the depression of minute volume induced by co-administration of morphine and saline (Fig. 6.12G). However, co-administration of morphine, pregabalin, and ethanol did not depress minute volume significantly more than morphine co-administered with pregabalin in high oxycodone pump-implanted mice. Additionally, the degree of minute volume depression by co-administration of morphine, pregabalin, and ethanol was still significantly diminished compared to saline pump implanted controls (Fig. 6.12G).

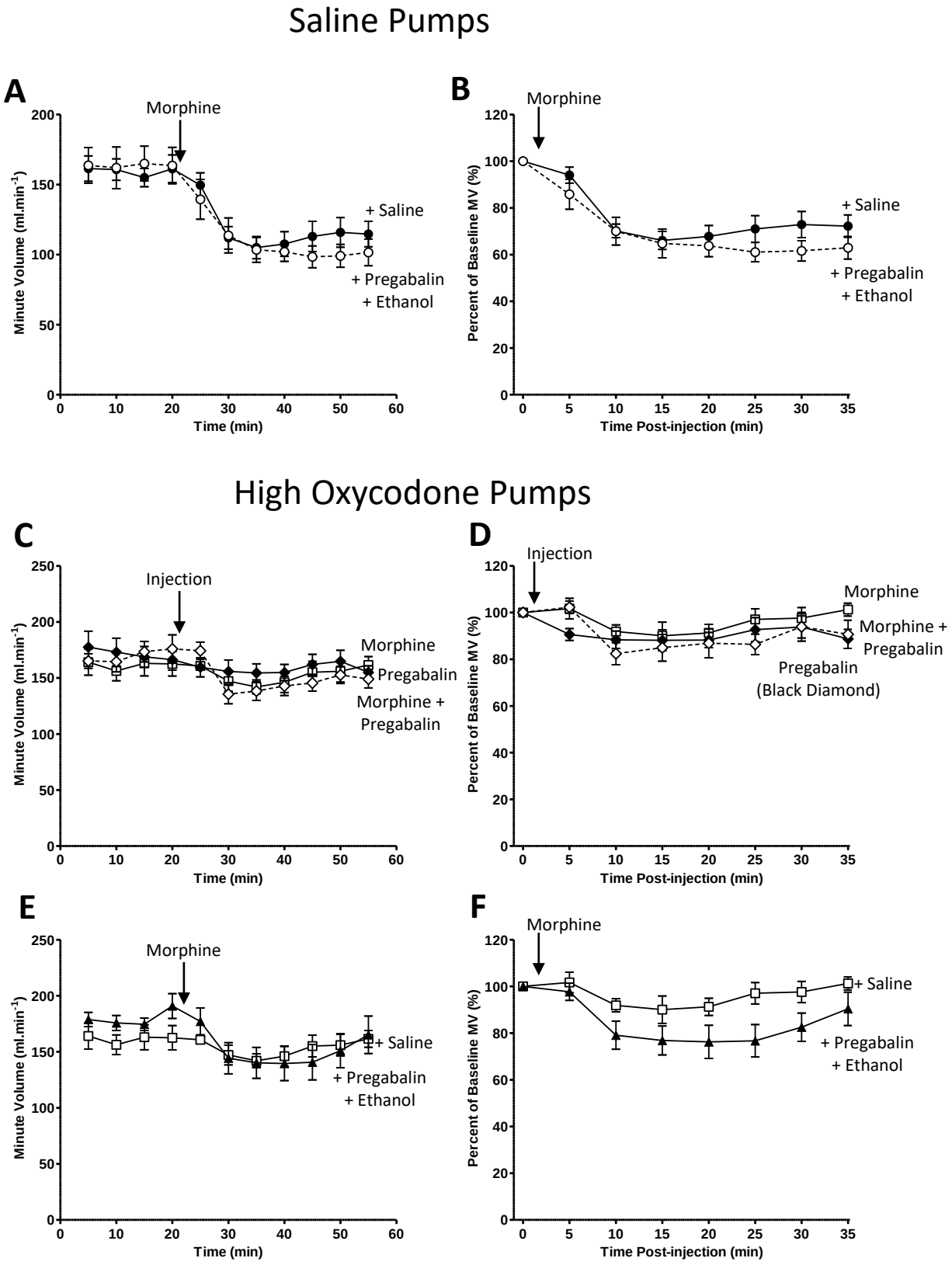


Figure 6.12A-F: Effect of Acute Pregabalin on High Oxycodone induced tolerance to morphine respiratory depression. (**A-B)** Co-administration of ethanol (0.3 g/kg), pregabalin (20 mg/kg) and morphine (10 mg/kg) did not enhance the decrease in MV compared to control. (**C-D**) 6d high oxycodone pump implanted mice injected with pregabalin and saline or pregabalin and morphine at the same time did not cause a change in MV. (**E-F**) Co-administration of pregabalin, ethanol, and morphine in high oxycodone pump mice did cause a significant decrease in mouse MV (**G**) N=7 for all groups.

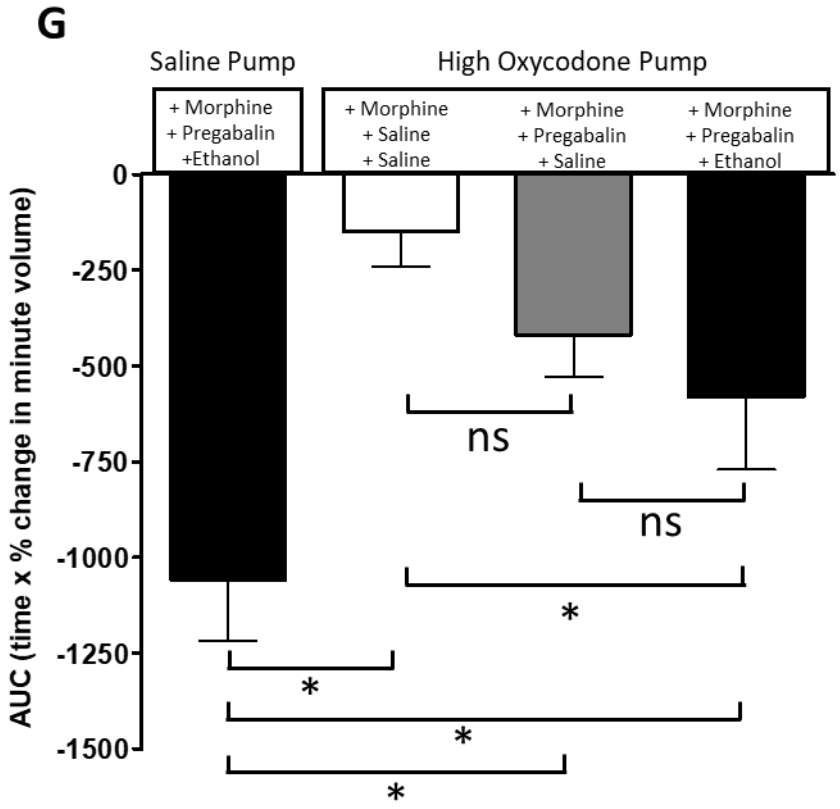


Figure 6.12G: Effect of Acute Pregabalin on High Oxycodone induced tolerance to morphine respiratory depression. The decrease in MV induced by morphine and saline co-administered to high oxycodone pump mice was significantly reduced from saline pump control. The co-administration of pregabalin and morphine did not significantly enhance the decrease in MV compared to morphine administered with saline in high oxycodone pump mice. Co-administration of pregabalin, ethanol and morphine did significantly enhance the decrease in MV seen compared to morphine administered with saline. The decrease in MV was significantly reduced compared to saline pump control. * indicates $p<0.05$ groups were compared with a One-way ANOVA followed by Bonferroni's comparison. N=7 for all groups.

6.5.4 Effect of Acute Pregabalin on Methadone Induced Tolerance to Morphine Respiratory Depression

As methadone is a prevalent opioid substitution therapy for opioid addiction (Hickman et al., 2018), and those service users undergoing substitution therapy are known to experience anxiety during this process and in general (Milby et al., 1996), it is likely that pregabalin may be prescribed to methadone-maintained users in order to treat their symptoms of anxiety. As such this population may be particularly susceptible to accidental overdose following consumption of pregabalin and opioids.

Therefore, morphine was also co-administered with pregabalin in methadone pump-implanted mice to investigate the ability of pregabalin to reverse methadone induced tolerance to morphine respiratory depression. Male CD-1 mice received a prolonged treatment of either methadone or saline through implantation of an osmotic mini-pump for 6 d (See Materials and Methods section 2.4.1).

Morphine co-administered with saline in saline pump-implanted mice depressed minute volume (Fig. 6.13A-B). Morphine (10 mg/kg) co-administered with saline in methadone pump-implanted mice did not depress minute volume (Fig 6.13A-B) and this was significantly reduced compared to the depression of minute volume induced by morphine and saline co-administration in saline pump-implanted mice.

The co-administration of morphine and pregabalin (20 mg/kg) in methadone pump-implanted mice also did not depress minute volume (Fig 6.13A-B). With no significantly greater depression of minute volume compared to morphine and saline co-administered in methadone pump-implanted mice (Fig. 6.13C).

These data indicate, that like ethanol (Hill et al., 2016), pregabalin does not present an immediate risk to morphine tolerance that has been induced and maintained through methadone treatment.

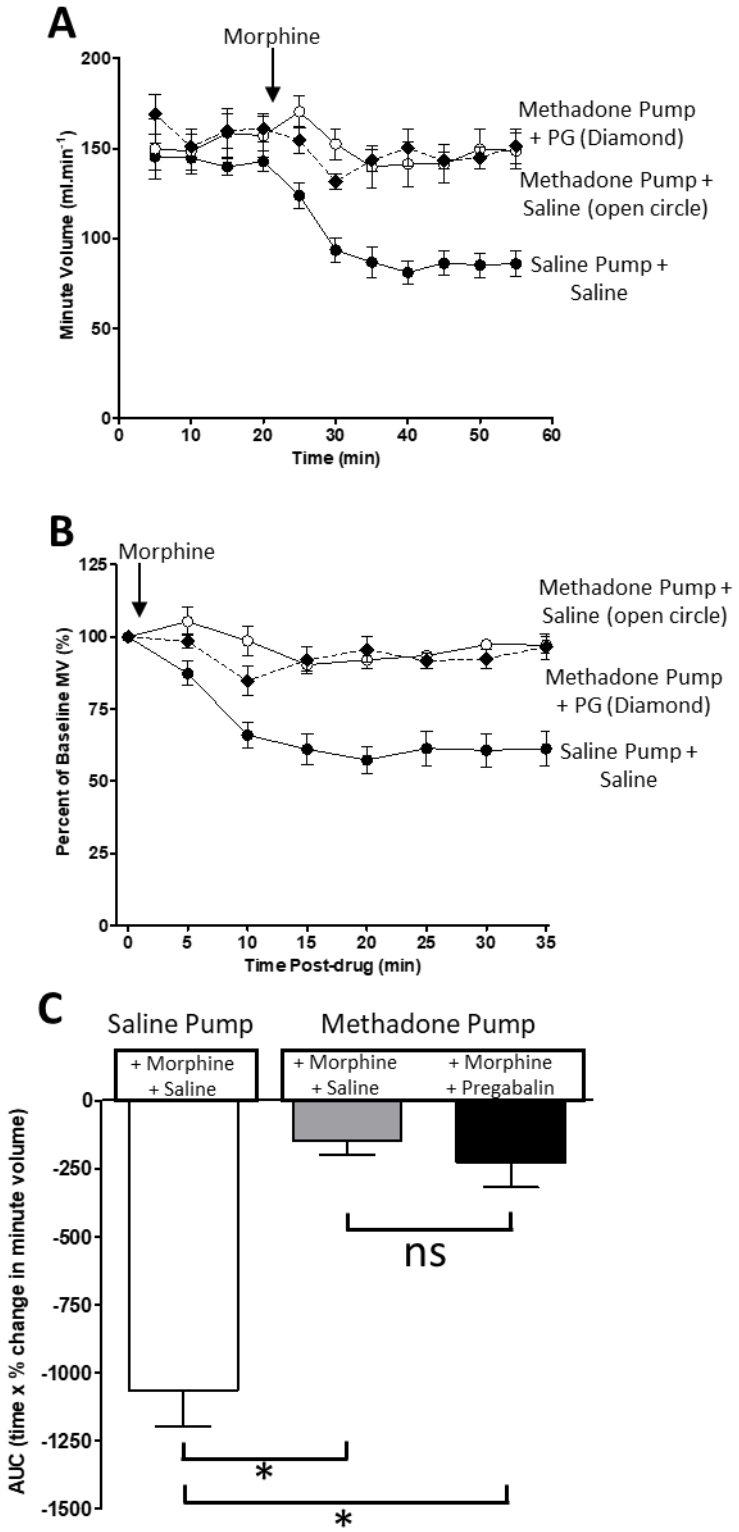


Figure 6.13: Effect of Acute Pregabalin on Methadone induced tolerance to morphine respiratory depression. (**A-B)** Co-administration of pregabalin (20 mg/kg) and morphine (10 mg/kg) did not enhance the decrease in MV compared to control in 6d methadone pump implanted. (**C**) Methadone pump treatment significantly reduced the decrease in MV induced by morphine. Pregabalin and morphine did not induced a significant decrease in MV compared to morphine alone and was still significantly reduced compared to control. * indicates $p<0.05$ groups were compared with a One-way ANOVA followed by Bonferroni's comparison. N=7 for all groups.

6.6 Discussion

6.6.1 Ethanol

Ethanol (as alcohol) is an extremely popular drug within many societies around the world. Ethanol is the most common drug of abuse used alongside opioids and the prevalence of ethanol abuse amongst opioid users is likely a reflection of how readily obtainable ethanol is compared to illicit drugs. In addition, ethanol is detectable post-mortem in at least 30% of all fatal opioid overdoses (Hickman et al., 2008b, Darke, 2003, Ruttenber et al., 1990).

The presence of ethanol in fatal opioid overdoses has been attributed to three potential explanations (Hickman et al., 2008b):

1. An opioid user intoxicated on ethanol is more likely to use a larger dose of opioid following ethanol induced inhibition of cognitive function.
2. The respiratory depressant activity of ethanol and opioid act in summation to produce a fatal degree of respiratory suppression.
3. Ethanol has activity that reduces the degree of opioid tolerance which in turns renders an otherwise innocuous dose of opioid now fatal.

If either the first or second options from the above were true, one would hypothesise in these instances that a significant blood concentration of either ethanol or opioid would be present. However, there is in fact an inverse correlation between post-mortem blood ethanol and blood morphine content in heroin opioid overdoses (Ruttenber et al., 1990) with a cluster of opioid overdose deaths having low concentrations of both ethanol and morphine present (Chapter 1 Fig. 1.5). This would suggest that the first and second options are less likely to be responsible for the significant co-morbidity witnessed with ethanol and opioids as in these circumstances a positive correlation between ethanol and opioid blood concentrations would occur with both being consumed in large doses, or there would be a cluster of fatalities where at least one of ethanol or opioid would have a high blood concentration.

These results therefore supported investigation of the third option, that ethanol is able to reverse maintained tolerance in opioid users, converting a non-lethal dose of opioid into one that produces fatal respiratory depression. Previous work has provided evidence that acute ethanol reverses morphine-induced tolerance to morphine antinociception (Hull et al., 2013) and acute ethanol is also able to reverse morphine-induced tolerance to morphine respiratory depression (Hill et al., 2016). In addition, Hill et al (2016) also demonstrated that acute ethanol was unable to reverse tolerance to morphine respiratory depression that had been induced by methadone.

Previous research on the relative intrinsic efficacy of opioid agonists for G-protein versus GRK dependent signaling pathways demonstrated that methadone had a significantly greater intrinsic efficacy for GRK activation compared to that of morphine (McPherson et al., 2010). It has also been suggested that methadone desensitizes the MOPr through a GRK dependent mechanism and produces tolerance through a GRK dependent mechanism (Melief et al., 2010). In contrast there is significant evidence supporting morphine desensitization of the MOPr and morphine tolerance being dependent on G-protein signaling (Bailey et al., 2009a, Bailey et al., 2009b, Lin et al., 2012, Melief et al., 2010). If ethanol lowers morphine tolerance through inhibition of a G-protein dependent mechanism that maintains morphine tolerance, then this would explain the insensitivity of methadone tolerance to reversal by ethanol.

McPherson et al (2010) also identified oxycodone as having a low intrinsic efficacy for GRK activation, similar to that of morphine. From the results published in McPherson et al (2010), we hypothesised that oxycodone induced tolerance to morphine respiratory depression would also be susceptible to reversal by ethanol. Tolerance to morphine induced by medium oxycodone pump-implantation was in fact reversible by acute ethanol administration, suggesting that it is a low intrinsic efficacy for GRK activation that predicts whether tolerance induced by an opioid will be reversible by ethanol administration.

However, tolerance induced by high oxycodone pump-implantation was not reversible by ethanol, suggesting that at higher doses of oxycodone sufficient GRK is recruited to initiate GRK dependent mechanisms of tolerance at the MOPr. Additionally, there is evidence that morphine induces tolerance through a GRK dependent signaling (Bohn et al., 2000, Bohn et al., 2002). If both morphine and methadone induce tolerance at the MOPr through a GRK dependent signaling pathway then the relative sensitivity to reversal by morphine must occur through a separate mechanism.

However, previously (See Chapter 5 section 5.2 & 5.3.1), both morphine and medium oxycodone induced tolerance were reversible by PKC inhibition, indicating that tolerance induced by morphine and medium oxycodone must include a G-protein component, as PKC is exclusively recruited through G-protein activation. Given that high oxycodone induced tolerance also displays insensitivity to PKC inhibition, this does not rule out a dose dependent activation of GRK in the development of tolerance for both oxycodone and morphine.

6.6.2 Acetaldehyde

Acetaldehyde has previously been considered the innocuous metabolite of ethanol, responsible only for aversion following accumulation (Peana and Acquas, 2013). The reputation of acetaldehyde has undergone a rehabilitation in recent years, highlighting the possibility that acetaldehyde may be responsible for some key actions of ethanol. Chelation of acetaldehyde to an inactive state, whilst leaving ethanol metabolism unimpaired, has been shown to reduce ethanol consumption in rodents pre-disposed to drink ethanol (Font et al., 2006). The suggestion being that the rewarding properties of ethanol also rely on pharmacological actions of acetaldehyde.

Acetaldehyde has also been shown to producing conditioned placed preference in rodents (Spina et al., 2010) as well as produce self-administration in rodents that will continue to work for acetaldehyde through persistent foot shock negative reinforcement (Cacace et al., 2012). These rodents also then display relapse like behavior with acetaldehyde specific cue reinstatement. These results indicating that acetaldehyde is addictive, even in the absence of its precursor – ethanol.

Acetaldehyde dose dependently reversed morphine tolerance with full reversal seen at the highest dose. This result in conjunction with acetaldehyde chelation inhibiting ethanol reversal of morphine tolerance, suggests that ethanol reversal of morphine tolerance relies on the action of acetaldehyde in part at least. However, it is currently extremely difficult to measure the brain concentration of acetaldehyde produced following acute ethanol administration. Therefore, whilst the doses of acetaldehyde administered confirm acetaldehyde can reduce morphine tolerance, the relative contribution of acetaldehyde as a metabolic component of ethanol reversal of morphine tolerance is not fully understood.

6.6.3 Prolonged Ethanol Consumption

Acute ethanol reversal of morphine tolerance is a highly simplified animal model of ethanol polydrug abuse compared to the ethanol consuming habits of humans. In particular, ethanol is known to be consumed chronically by many opioid users (Maremmani et al., 2007). The chronic consumption of ethanol may therefore play an important role in the development or ongoing maintenance of morphine tolerance, or indeed alter the metabolism or distribution of morphine following heroin use.

The prolonged administration of ethanol to mice and implantation of morphine pumps does not mimic the polydrug consumption of humans, but it does allow investigation of ethanol and its action on the earlier stages of morphine tolerance. The fact that no appreciable tolerance develops to morphine in ethanol fed mice suggests that ethanol either prevents the development of tolerance, or that tolerance develops through a mechanism uninterrupted by ethanol, but this not maintained due to prolonged ethanol. Multiple stages of MOPr desensitization and tolerance following morphine binding have previously been suggested (Levitt and Williams, 2012) with a acute developing desensitization followed by tolerance. This may also be true for morphine tolerance following MOPr desensitization, however, the distinction between ethanol inhibiting the development or maintenance of morphine tolerance is not inferable from these results.

These data do however, demonstrate that this particular paradigm of prolonged ethanol exposure does not cause a change in morphine distribution between blood and brain and so the action of ethanol on morphine tolerance is likely due to ethanol inhibiting tolerance at the level of the MOPr. However, this prolonged ethanol paradigm is known to have limited pathology on metabolism in the liver (Bertola et al., 2013) and so this does not exclude pathology of morphine metabolism potentially playing an important role in ethanol and morphine co-morbidity in humans.

Overall, there is strong evidence to suggest that abuse of both heroin and ethanol over a prolonged period will reduce the safety margin between the desired euphoric effect of heroin consumption and the undesired, unintentional effect of heroin induced respiratory fatal overdose. The ability of acute ethanol to reverse medium oxycodone tolerance would suggest that prolonged ethanol would similarly increase the risk of oxycodone use, whereas prolonged consumption of methadone and ethanol is unlikely to see the same escalating risk.

6.6.4 Pregabalin

As pregabalin, and gabapentoids in general, emerge as drugs co-abused with opioids, and the rates of fatal overdoses involving gabapentoids escalates, it is important to examine the possibility of additional interaction between gabapentoids and opioids that goes beyond summation.

Pregabalin reversed tolerance to morphine induced by morphine or medium oxycodone treatment but not tolerance induced by high oxycodone treatment. Pregabalin and ethanol share a pattern of reversing morphine and medium oxycodone tolerance but not high oxycodone tolerance, additionally, ethanol and pregabalin co-administered did not cause further reversal of high oxycodone tolerance. This would suggest some commonality in their mechanism of action perhaps, given that if pregabalin and ethanol acted at separate mechanisms to reverse tolerance at the MOPr, co-administration would likely reveal a summation of these effects.

Pregabalin is known to bind α2-δ, an auxiliary subunit of voltage-gated calcium channels, and in doing so reduce synaptic neurotransmitter release (Taylor et al., 2007). Ethanol is also known to interact with voltage-gate calcium channels, specifically L-type calcium channels (Mah et al., 2011, Pietrzykowski et al., 2013). The role that calcium channels might play in morphine induced tolerance at the MOPr is not fully understood, though inhibition of N-type voltage-gated calcium channels does occur following MOPr agonist activation and is mediated by the G-protein signaling pathway, specifically the β/γ-subunit. Potentially the actions of both ethanol and pregabalin on opioid tolerance may relate to the ability of each to interact with voltage-gated calcium channel activity.

6.6.5 Conclusion

In conclusion, this chapter demonstrated that both ethanol and pregabalin are able to fully reverse tolerance to morphine respiratory depression that has been induced by morphine or medium oxycodone, but only partially or not at all when induced by high oxycodone and methadone respectively. Additionally, this chapter demonstrated that ethanol is not only able to reverse morphine induced morphine tolerance but also to prevent its development or maintenance, and that the primary metabolite of ethanol acetaldehyde, may in fact be a key mediator of ethanol reversal of opioid tolerance, rather than just ethanol alone.

7.0 General Discussion

7.1 Introduction

The main aims of the work described in this thesis were to investigate the development of tolerance to opioid-induced respiratory depression (OIRD), the mechanisms of tolerance to OIRD, and how polydrug abuse may affect tolerance to OIRD. These questions were investigated across multiple opioid agonists that are of clinical relevance, varying efficacy and varying potency. This approach was intended to provide a composite view of tolerance to OIRD wherein similarities and differences between opioid agonists may allow conclusions on general mechanisms of tolerance to be inferred.

Opioids are still considered the gold standard for pain therapy. Yet the use of opioids is hindered by the development of tolerance, their propensity to induce euphoria leading to addiction, and their ability to depress respiration. The depression of respiration by opioids is the major cause of death following opioid overdose (White and Irvine, 1999), therefore developing an understanding regarding the likelihood of developing tolerance to OIRD, a knowledge of tolerance mechanism(s) and an understanding of commonly consumed drugs that may alter developed tolerance is crucial.

Not only will this inform prescription and care practises to help best prevent avoidable fatal opioid overdose incidences, but indeed understanding the underlying cause of tolerance may ultimately allow the development of an opioid that does not suffer from this flaw, and therefore prevents dose escalation.

7.2 Tolerance Develops to Opioid Respiratory Depression

There is considerable interest in investigating the potential development of tolerance to OIRD (Ayesta and Florez, 1990, Brandt and France, 2000, Dumas and Pollack, 2008, Hayhurst and Durieux, 2016, McGilliard and Takemori, 1978, Paronis and Woods, 1997). Multiple publications have discussed an absence of tolerance to OIRD following opioid treatment using *in vivo* experiments (Kishioka et al., 2000, Ling et al., 1989, Brandt and France, 2000, McGilliard and Takemori, 1978, Paronis and Woods, 1997); results that are inconsistent with results presented in this thesis. There are key differences in experimental protocol to account for these discrepancies. Primarily the difference lies in the length of exposure to the opioid agonist.

Previous work (Ling et al., 1989) utilised extremely short administration of an opioid (8 hr) to investigate the development of tolerance to OIRD, in which no tolerance was found. However, one must consider that this compares to a 6-day administration of opioid in the experimental protocols of this thesis, representing approximately 18 times the length of administration. A single dosing treatment to examine tolerance to respiratory depression is not particularly representative of opioid administration habits utilised either in a clinical setting or in an opioid abuse setting. As tolerance in this thesis was assessed a full 6-days after initiation of opioid treatment, it is entirely possible that an acute challenge dose of morphine administered 8 hr after the beginning or prolonged opioid treatment would elicit a normal depression of respiration. The results presented in Ling et al, is therefore not therefore considered contradictory with that presented in this thesis.

Additional investigation of tolerance to OIRD utilised intermittent injections of heroin over an extended 3-day period (Kishioka et al., 2000) that did not induce tolerance to OIRD. That this did not show a development of tolerance may also be indicative of its relatively short time frame, given that previously published work from my MSc research showed that the development of tolerance to OIRD took a full 5-days of opioid administration. Equally however, this may be indicative of the dosing schedule used for tolerance induction. Previous work has described a greater extent of tolerance is developed to opioidergic effects following continuous administration of an opioid compared to intermittent access (Dighe et al., 2009).

Primarily Dighe et al (2009) conclude that sustained release formulations of opioids for the management of pain are more likely to induce tolerance compared to intermittent periodic dosing. Dighe et al (2009) speculate this may relate to phasic receptor activation that allows recovery of downstream events related to the development of tolerance.

This represents the most likely difference in protocol between Kishioka et al and other intermittent dosing protocol papers (McGilliard and Takemori, 1978, Paronis and Woods, 1997) and the data presented within this thesis. This may account for the difference in results regarding the development of tolerance (or lack thereof) to OIRD.

There is no specific attempt within the published literature referenced (McGilliard and Takemori, 1978, Paronis and Woods, 1997, Dighe et al., 2009, Ling et al., 1989) or within this thesis, to explicitly model the human situation regarding opioid abuse and the development of tolerance to OIRD. The experiments conducted within this thesis and past work are models of tolerance that investigate the potential for tolerance to occur and the potential mechanisms involved in tolerance. Intermittent or continuous forced administration of opioid to rodents or primates cannot replicate the self-administration of opioid by human opioid users.

Determination of the opioid dose inducing overdose in humans is difficult due to the polydrug abuse situation most opioid users inhabit, there are relatively few incidences of opioid overdoses, fatal or otherwise, where other depressant drugs such as alcohol or benzodiazepines are not also present (Mathers et al., 2013, Schifano et al., 2018, Meacham et al., 2016, Lyndon et al., 2017). However, there is also evidence to suggest that increasing rates of opioid prescription or opioid use correlate with an increased opioid-based mortality rate (Kolodny et al., 2015, Okie, 2010). There is also an increase in the likelihood of overdose following the immediate initiation or cessation of opioid substitution therapy (Bird et al., 2016, Strang et al., 2003, Darke and Hall, 2003). This indicates that there may be differences in cross-tolerance between the abused and substituted opioids causing a vulnerability to overdose during initiation of therapy, or that tolerance declines during the tapering of therapy, therefore increasing the risk of fatal overdose following cessation of treatment. These human studies suggest that tolerance to OIRD in an *in vivo* experiment may strongly depend on the opioid used as well as the temporal nature of its administration as well as the specific measure by which tolerance is used e.g. is the challenge opioid the same or different to the induction opioid.

Whilst the experimental protocol within this this thesis does not directly attempt to reconstitute the human condition, the results nonetheless prove that in the correct conditions, the development of tolerance to OIRD is possible.

7.3 Methadone Induces Tolerance to Morphine Respiratory Depression

Methadone is commonly prescribed for opioid substitution therapy (OST) (Darke and Ross, 2001). Opioid users prescribed methadone for OST commonly receive methadone once daily via oral consumption (Hickman et al., 2018). Once daily is sufficient for OST practise as the half-life of methadone is prolonged in humans (Glue et al., 2016) with effective blood concentrations of methadone to a minimum of 18 hours after consumption of methadone.

Given that prolonged methadone treatment in mice induces tolerance to morphine respiratory depression, and that morphine is the major active metabolite of heroin, these data would suggest that methadone is an effective OST in aiding the prevention of fatal opioid respiratory depression following relapse in methadone treated opioid addicts.

However, the dose of methadone administered in these experiments may constitute an extremely high relative dose of methadone in humans, as the half-life of methadone in mice is much lower than in humans (Beauverie et al., 1994) and so this was compensated for in this thesis through administration of a high dose of methadone. Considerable variation in the dose of methadone for OST is known to occur, as individual's metabolism of methadone is known to vary substantially (Glue et al., 2016), therefore high and low dosing of methadone in humans is relative to each individual user. Overdose deaths in methadone maintained opioid users is known to occur (Faul et al., 2017, Kimber et al., 2015) and though the results in this thesis suggest that on top use of heroin during methadone OST would be less likely to produce fatal respiratory depression, this may only be true of opioid users maintained on high doses of methadone.

A low maintenance dose of methadone may be insufficient to induce protective tolerance to on top heroin use, which may therefore account for the rate of deaths that occur in the methadone-maintained opioid using population. The low maintaining dose of methadone has persisted so long, historically, due to a misinterpretation of early pharmacokinetic data, suggesting a dosing range of 30-60 mg daily would be sufficient in methadone maintenance. However, recent guidelines have altered the recommended range of methadone to be increased to 60-120 mg daily (DoH-UK, 2017), which is more likely to provide a significant block of on top opioid use.

As prolonged buprenorphine administration has previously been shown to prevent morphine respiratory depression in mice (Hill et al., 2015) and human studies have demonstrated up to an 80% reduction of on top overdose deaths in buprenorphine maintained opioid users (Auriacombe et al., 2004), buprenorphine may constitute a more effective form of OST for opioid users. Buprenorphine is not thought to protect from on top heroin use through induction of tolerance, but through maintained low efficacy, high affinity binding to the MOPr orthosteric site, preventing the binding of heroin. Additionally, buprenorphine has an even more prolonged half-life than methadone (Chiang and Hawks, 2003), therefore intermittent dosing with a receptor saturating concentration of buprenorphine should be sufficient to produce significant blockade of on top heroin use.

Methadone maintenance treatment may require specific titration of doses to opioid users over a prolonged period e.g. 12-18 months, in order to administer the most effective dose at generating protective tolerance to on top heroin use. This conclusion contrasts with current NICE/NHS guideline that only suggest a mandatory maintenance period on methadone for 3 months as a transitionary period for opioid users to achieve full abstinence (Guidelines, 2007). This may prove to be ultimately counter-productive guidelines for the administration of methadone, resulting in more effective on top use of heroin inducing significant relapse or unintended and potentially fatal overdoses.

However, the most recent released guidelines have, in light of recent research and conclusions such as these presented here, have suggested that the most effective prescribing methods to prevent overdose whilst undergoing OST and to effectively manage withdrawal symptoms, thus resulting in the most positive outcome for the opioid user, is to combine the prescription of buprenorphine and methadone in a combined pharmacotherapy (DoH-UK, 2017).

7.4 Oxycodone Induces Tolerance to Morphine Respiratory Depression

Oxycodone is a commonly abused prescription opioid in the USA (Hedegaard et al., 2017a). It has become increasingly common, due to the prescription culture of the USA (Elbe et al., 2015), for patients with legitimate need for oxycodone therapy due to pain to become addicted to the euphoric effects of oxycodone. Additionally, there is a significant population of oxycodone abusers who obtained oxycodone from diverted legitimate prescriptions of oxycodone (Cicero et al., 2011, Inciardi et al., 2010), due to a prevalence for over prescription of oxycodone to pain patients (Elbe et al., 2015).

However, due to the supply of oxycodone to both of these population relying on a legitimate prescription at some point, either for direct consumption or diverted consumption of oxycodone, the stability of oxycodone supply is relatively poor compared to that of heroin (Mars et al., 2014, Cicero et al., 2014). This is thought to drive heroin consumption once oxycodone is no longer available due to an inability to procure oxycodone on, or diverted from, a prescription (Cicero et al., 2014).

However, there is evidence to suggest that there are problems in both opioid prescribing and opioid using population in correctly ascertaining the dose required, be that for pain relief or euphoria (CDC, 2014, Lankenau et al., 2012, Frank et al., 2015). In this case a dose of heroin far more than the previously used oxycodone dose may be able to overcome any cross-tolerance and induce fatal respiratory depression.

Whilst oxycodone is prescribed for the management of pain, there is evidence that indicates the majority of the population that is oxycodone dependent and therefore abusing oxycodone is in fact procuring oxycodone illicitly from street vendors of the drugs (Cicero et al., 2011, Inciardi et al., 2010) rather than legitimate pain patients abusing a prescription. Though there is also considered a high proportion of legitimate prescriptions that are diverted from pain patients due to the profitability of doing so.

The population of oxycodone dependent users who subsequently switch to heroin are thought to almost exclusively arise from the illicitly bought street oxycodone population, with reasons often cited as ease of access to street heroin and the relatively low price of street heroin compared to formulation tablets of oxycodone (Ciccarone, 2009, Dasgupta et al., 2013).

The increase in heroin overdose deaths seen since 2010 (Fig. 7.1) (Compton et al., 2016) may well be indicative of crossover populations of opioid users, as ongoing oxycodone dependent population switches to heroin. Indeed, over this same period it has been noted that the rate of increase in semi-synthetic opioid overdose deaths (predominantly oxycodone) has slowed and begun to level off, though still remaining high (CDC-Wonder, 2017).

The extent to which an oxycodone dependent user will have cross-tolerance to heroin following a switch in abused opioid would appear to depend on the dose of oxycodone currently used. There is clear evidence in this thesis or differences in both the level of tolerance to morphine obtained from prolonged oxycodone use as well as the mechanisms of tolerance, which may also be indicative of how susceptible an oxycodone user will be to polydrug overdose with heroin and ethanol.

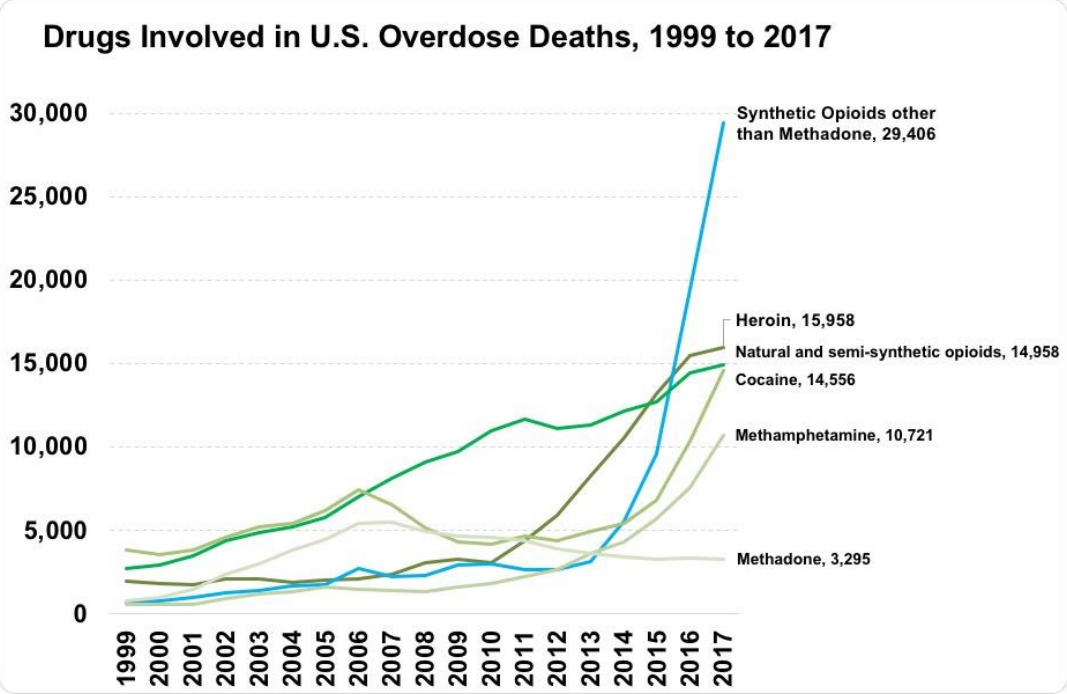


Figure 7.1*: Drugs Involved in U.S. Overdose Deaths* *- Among the more than 72,000 drug overdose deaths estimated in 2017, the sharpest increase occurred among deaths related to fentanyl and fentanyl analogues (synthetic opioids) with nearly 30,000 overdose deaths. Source: (CDC-Wonder, 2017)*

7.5 Tolerance to OIRD as a Consequence of the Experimental Protocol Used

As previously discussed (See Discussion section 7.2) tolerance to OIRD in mice may occur due to both the length of opioid administration as well as the specific means of administration. In summary, continuous administration of opioid over a prolonged period appears more likely to result in tolerance to OIRD compared to intermittent administration of opioids, lower doses of opioids or both factors combined. Most prescribed opioids are intended for intermittent administration (Schneider et al., 2003), though in the case of methadone and buprenorphine, prolonged opioid exposure does occur. Additionally, the consumption habits of opioid users are not one of continuous, but intermittent administration of opioids (Garland et al., 2013).

Considering the dissonance between experimental administration of opioids in mice compared to the administration of opioids in a clinical or addictive context, the relevance of tolerance to OIRD in mice to human opioid tolerance needs to be considered. Tolerance to OIRD in mice may arise simply as a consequence of the dosing protocol used, which may represent a pharmacological event that is unlikely to ever occur in humans.

Opioid abusers are very often engaged in opioid use over extremely long periods of time, often exceeding 10 years (Darke et al., 2002b, Hall and Darke, 1998, Ruttenber et al., 1990). When one examines the social aetiology of overdosing opioid addicts, there is a significant proportion of fatal overdoses occurring in opioid addicts that have recently undergone a period of de-toxification from opioid use (Ruttenber et al., 1990). Crucially, the amount of opioid administered in these cases is thought to represent a dose of opioid (often heroin) that is lower than commonly administered in living addicts (Brewer, 2002). From this, it could be surmised that opioid abstinence has led to a significant decay in tolerance to OIRD, such that a previously non-lethal dose has resulted in fatal overdose. Logically, for this assertion to hold true, there must indeed be a development of tolerance to OIRD in opioid addicts for opioid abstinence to present the clear opioid overdose risk that occurs on resumption of opioid abuse.

In addition to these conclusions, there are other parallels with results generated in mice to result collected from humans. A large population study in France found that buprenorphine substitution treatment for heroin addicts achieved an 80% reduction in incidences of overdose when on top use of heroin occurred (Auriacombe et al., 2004). This result matches well with mouse experimental results that 6 d buprenorphine treatment prevents the depression of respiration by acute morphine (Hill et al., 2015), supporting the conclusion that the underlying pharmacology is similar in both of these situations.

However, there is also evidence that methadone-maintained users are competently aware of the half-life of methadone following oral administration, and careful timing does allow for effective on top use of heroin in methadone-maintained heroin users following perceived clearance of methadone in approximately 18 hr (Lyndon et al., 2017). This would suggest that whilst continuously dosed methadone induces tolerance in mice, the relatively intermittent nature of methadone administration in humans that allows a brief window of effective on top heroin use is not comparable to the mouse experimental model.

These results would also suggest that in terms of opioid substitution therapies, receptor blockade by buprenorphine is a more effective strategy for preventing overdose than the use of methadone to maintain opioid users and generate tolerance to OIRD. Though as previously discussed (Section 7.3), the recommendation in the current prescribing guidelines in the UK is to manage OST through a combination of methadone and buprenorphine pharmacotherapies (DoH-UK, 2017).

7.6 Opioids and Polydrug Abuse: Ethanol, Cocaine and Benzodiazepines

Whilst ethanol is the most commonly abused drug with opioids (Darke and Hall, 2003, Ruttenber et al., 1990, White and Irvine, 1999), this is likely due to ethanol being freely available to purchase in many countries around the world, as alcohol; once the legal age has been reached for a given country. Undoubtedly this availability plays a role in the prevalence of alcohol as an additional drug of abuse.

However, both benzodiazepines and cocaine are also very common additional drugs of abuse administered alone with opioids in a polydrug scenario (Schifano et al., 2018, Zoorob, 2018). Benzodiazepines are very commonly found post-mortem following fatal opioid overdose, however, benzodiazepines and opioid overdose deaths are distinct from ethanol and opioid overdose deaths as there is a positive correlation between the residual blood levels of opioid and benzodiazepine (Steentoft et al., 1996, Kerr et al., 2007, Park et al., 2015) suggesting a summation of CNS depression leading to fatal respiratory depression. Whereas ethanol blood concentration in ethanol-opioid fatal overdose incidences has a very well defined inverse correlation with the blood concentration of opioid (Ruttenber et al., 1990).

Post-mortem examination of cocaine blood levels in fatal opioid overdose does not appear to have any definable correlation between the detected concentration of cocaine and the detected concentration of morphine (Minett et al., 2010), however this may be because cocaine and heroin are very often abused with additional drugs such as benzodiazepines, ethanol or antidepressants (Minett et al., 2010) that actually form the lethal combination of polydrug abuse.

Ethanol and opioid polydrug abuse is unique amongst the three most commonly abused drugs alongside opioids, due to the inverse correlation in blood levels witnessed. Benzodiazepine and cocaine polydrug abuse with opioids is still an incredibly important matter of public health interest due to the increase in the likelihood of fatal overdose (CDC-Wonder, 2017), but there is not a great amount of evidence to suggest an interaction between benzodiazepines or cocaine with the inherent mechanism of opioid tolerance at the level of the MOPr.

However, previous research (Fields et al., 2015) and recently reported data at the International Narcotics Research Conference 2018 does suggest that diazepam specifically inhibits the primary cytochrome P450 enzyme (CYP3A4) responsible for oxycodone metabolism. This suggests that co-abuse of diazepam and oxycodone may significantly potentiate the effect of oxycodone by inhibiting the clearance of oxycodone and therefore increase the risk of fatal respiratory depression by oxycodone.

7.7 Ethanol Reversal of Tolerance

Previous results have indicated that not only is ethanol able to reverse desensitization of the MOPr in isolated rat brain slices (Llorente et al., 2013), but also dose-dependently reverse morphine and oxycodone induced antinociceptive tolerance (Hull et al., 2013, Jacob et al., 2017). These results suggest that ethanol administration is able to disrupt the maintenance of ongoing tolerance at the level of the MOPr.

That results presented in this thesis have shown that acute ethanol is able to reverse established tolerance to morphine, induced by both morphine and oxycodone. Additionally, prolonged ethanol is able to prevent the formation of tolerance to morphine induced morphine tolerance. These results again suggest that ethanol is able to interfere with the maintenance of ongoing tolerance, and potentially is able to disrupt the development of tolerance.

These results agree with the general notion that concomitant consumption of alcohol and opioids pre-disposes an addict to a greater likelihood of an overdose incident (Hall and Darke, 1998, Hickman et al., 2008b, Hull et al., 2013, Ruttenber et al., 1990). However, previous discussion regarding the co-morbidity of ethanol and heroin has resulted in several suggestions as to why this mix of drugs is particularly lethal (See Chapter 6 section 6.6.1). In summary, the depressant action of ethanol and heroin may summate to produce fatal respiratory depression; the cognitive inhibition from ethanol consumption may result in accidental overdosing with heroin; or ethanol may reduce established tolerance to heroin induced respiratory depression, rendering a dose previously considered innocuous, now fatal.

The co-morbidity of ethanol and heroin is also confounded by the inverse correlation of ethanol and morphine blood levels in post-mortem analysis, with a significant cluster of deaths occurring with both low levels of ethanol and morphine (See Chapter 1 Fig. 1.5). In fact the concentration of ethanol in the majority of co-morbid deaths had previously been demonstrated to not induce significant depression of respiration (Gilliam and Collins, 1982), and a large proportion of deaths contained concentrations of morphine known to be lower than that seen in living users not experiencing overdoses (Brewer, 2002). The low concentrations of ethanol and morphine that had resulted in fatal respiratory depression, predominantly in more experienced users, was the catalyst for investigating the potential of ethanol to interact with opioid tolerance.

The results of this thesis in conjunction with preceding published results suggest that the co-morbid tendency of ethanol and heroin abuse together is most likely to result from ethanol reducing tolerance, rather than a summation of depressant effect or overdosing due to impaired judgement. Though there is not a parallel body of results examining ethanol and oxycodone concentrations post-mortem in the case of fatal oxycodone overdoses, the results within the preceding thesis indicate that ethanol is likely to induce a similar reduction in oxycodone-induced tolerance as that seen with morphine.

7.8 PKC Inhibition Reversal of Tolerance

There is currently a significant amount of research suggesting an involvement of PKC and the maintenance of tolerance to morphine (See Chapter 1 section 1.15). Previous work has shown that desensitization of the MOPr by, but not by DAMGO, is prevented by inhibition of PKC in both rat brain slices (Bailey et al., 2009a, Bailey et al., 2009b) and HEK293 cells (Johnson et al., 2006). Although in experiments conducted in rat brain slices, pre-activation of PKC through addition of a phorbol-ester was required to see an effect on morphine desensitization. However, constitutively active PKC isoforms expressed in mice, vastly accelerated the onset of tolerance to morphine antinociception, respiratory depression and constipation (Lin et al., 2012).

(Doll et al., 2011, Feng et al., 2011). Previous work has found that PKC inhibition reduces basal phosphorylation of the MOPr (Johnson et al., 2006), suggesting that in the non-agonist activated state there is interaction between PKC and the MOPr. Further work has demonstrated that PKC isoforms (α, δ, ε) are able to directly phosphorylate residues on the MOPr C-terminus tail (Doll et al., 2011, Feng et al., 2011) with two putative sites suggested for direct PKC phosphorylation of the MOPr, Ser363 and Thr370. Developing this area, it was confirmed that PKC specifically phosphorylated Ser363 on the MOPr C-terminus tail (Chen et al., 2013) with purified PKC using N-terminal glutathione S transferase (GST) fusion proteins. Though CamKII was found to phosphorylate Thr370 rather than PKC.

Previous research has highlighted the importance of the PKCα isoform in relation to morphine desensitization (See Chapter 1 section 1.15). In particular, previous work demonstrated that PKCα inhibition prevented morphine induced desensitization of the MOPr whereas PKCδ and PKCε inhibition did not (Bailey et al., 2009a). Additionally, morphine application to brain slices of the locus coeruleus from PKCα knock-out mice did not elicit desensitization of the MOPr. Furthermore, expression of constitutively active PKCα in mice dramatically enhanced the onset of tolerance to morphine antinociception, respiratory depression and constipation (Lin et al., 2012).

Exploring the converse of Lin et al (2012), PKCα knock-out mice were used within this thesis to examine the role of PKCα in the tolerance to morphine respiratory depression. These demonstrated that prolonged morphine is unable to produce tolerance to morphine respiratory depression was absent in PKCα knock-out mice. This result suggests that PKCα is recruited early on in either the development of tolerance to morphine when induced by morphine, or in the maintenance of tolerance to morphine.

The results in this thesis demonstrated that PKC inhibition is able to acutely reverse morphine tolerance, induced by morphine and oxycodone. Methadone induced morphine tolerance however, was not reversible by PKC inhibition. Previous work has established that morphine and oxycodone have similar, low, intrinsic efficacies for the activation GRK, whereas methadone has a high intrinsic efficacy for the activation of GRK (McPherson et al., 2010). This suggests that the mechanism of opioid tolerance likely to be recruited by an opioid agonist may be predicted by comparison of the relative intrinsic efficacy of an opioid agonist for G-protein versus GRK signalling. To provide stronger evidence to support this predictive measure, more opioid agonists presented within McPherson et al (2010) would have to be used to induce tolerance to morphine and compare the relative ability of PKC to reverse tolerance induced by each agonist.

7.9 Ethanol and the Inhibition of PKC

Previously published work has demonstrated that morphine desensitization of the MOPr, as measured by a decline in GIRK currents of the locus coeruleus in rats following application of morphine, can be enhanced by the addition of a PKC activator such as oxotremerine-M or a phorbol-ester (Bailey et al., 2004). Unpublished work from the laboratory has demonstrated that PKC activation enhanced morphine desensitization can be partially reversed by bath application of ethanol. However, in these experiments, the reversal of morphine desensitization was relatively low (<30% reversal) and the concentration of ethanol was 100 mM. At this concentration, ethanol is known to have a plethora of actions at multiple sites (See Chapter 1 section 1.13) and would represent significant intoxication if found in a human. Additionally, ethanol was not able to impact morphine desensitization in the absence of PKC activation.

The results in this thesis demonstrate a striking resemblance between ethanol dependent reversal of morphine and oxycodone tolerance and PKC inhibition dependent reversal of morphine and oxycodone tolerance. However, whilst these results are correlated, there is currently little direct evidence to demonstrate that ethanol directly or indirectly inhibits PKC.

One potential reason for the difference between the potent ability of ethanol to reverse morphine tolerance *in vivo* and relative inability to reverse morphine desensitization *in vitro* is ably demonstrated by the results obtained with acetaldehyde. Direct administration of acetaldehyde was able to reverse morphine tolerance, and previous work has established that ethanol reversal of morphine tolerance can be significantly inhibited by chelation of acetaldehyde by D-penicillamine.

Whilst the conversion of ethanol to acetaldehyde is known to occur *in situ* within the brain (Correa et al., 2008), the rate or abundance at which this might occur in a brain slice is not known, if it occurs at all. Potentially, the relative inability of ethanol to reverse morphine induced desensitization in rat brain slice electrophysiology may be indicative of the absence of acetaldehyde. If metabolism of ethanol is perturbed in a brain slice preparation, or indeed if the flow rate of solution across the brain slice prevents the accumulation of acetaldehyde to a concentration where it may impact on MOPr signalling, then this may prevent ethanol inhibiting morphine desensitization.

7.10 Gabapentoid and Opioid Abuse

The gabapentoids, gabapentin and pregabalin, have evolved from drug treatments primarily prescribed for epilepsy and neuropathic pain into, common drug treatment for multiple conditions, pre-dominant amongst these being anxiety, a condition that frequently occurs in heroin users. Gabapentoids have, until recently, been considered a safe alternative to opioid analgesics in conditions where the efficacy of opioids to produce analgesia is limited, such as neuropathic pain. Gabapentoids were thought safe due to a consensus that it was they were not liable to abuse and hard to overdose with (Gomes et al., 2017). This in turn has led to a relatively steady increase in the availability of pregabalin and gabapentin for those with the desire to take them in a non-therapeutic manner (Schjerning et al., 2016, Smith et al., 2016).

Recent research has indicated that both pregabalin and gabapentin are abused alone and in combination with other substances, including opioids (Bastiaens et al., 2016, Grosshans et al., 2013, McNamara et al., 2015, Smith et al., 2015). The incidence of opioid overdose deaths that involve gabapentin and pregabalin has risen substantially in the UK (Lyndon et al., 2017). There are more incidences of opioid abuse with gabapentin in Scotland compared to the remainder of the UK (ISD-Scotland, 2016), where pregabalin abuse with opioids is more common (Lyndon et al., 2017), however this is thought to reflect the relative level of prescription rates for each drug in Scotland, England, Wales and Northern Ireland respectively (Ruscitto et al., 2015, ISD-Scotland, 2016). This may indicate a summation of CNS depression by opioids and gabapentoids that induces lethal respiratory depression, or it may indicate a more nuanced interaction between gabapentoids and opioids.

That a low dose of pregabalin, devoid of intrinsic respiratory depression was able to reverse morphine tolerance induced by both morphine and oxycodone, would suggest that summation of depressant activity by opioids and pregabalin is not responsible for the co-morbidity of these drugs in humans. Additionally, the same low dose of pregabalin did not enhance acute morphine or oxycodone respiratory depression, suggesting that pregabalin does not inhibit the metabolism of morphine and oxycodone or alter the relative distribution of morphine and oxycodone, each of which could enhance opioid induced respiratory depression.

Therefore, this result indicates that pregabalin is likely to interact with tolerance to morphine either at the level of the MOPr or at the level of the immediate intracellular signalling pathways recruited by MOPr activation. However, pregabalin was unable to reverse tolerance to morphine induced by methadone, suggesting that pregabalin interaction with the MOPr or interaction with MOPr recruited signalling pathways is dependent on the opioid agonist used to induce tolerance.

This result bears a striking similarity to ethanol reversal of tolerance to morphine induced by morphine and oxycodone but not that by methadone. Indeed, the co-administration of ethanol and pregabalin did not result in an enhanced reversal of oxycodone tolerance, suggesting commonality on the mechanism of action for ethanol and pregabalin on oxycodone induced tolerance to morphine.

The implication from this result is that methadone maintenance may provide a relatively greater degree of protection for heroin users that use on heroin and pregabalin on top of a methadone treatment. Due to the structural and function similarity of pregabalin and gabapentin (Tzellos et al., 2010), it seems logical that gabapentin would provide the same reversal of opioid tolerance as pregabalin and as such methadone maintenance treatment may provide relative safety for those using either of these gabapentoids along with heroin. However, investigation of gabapentin-opioid use compared to pregabalin-opioid use must still be undertaken despite the similarities of gabapentin and pregabalin.

The amount of pregabalin abused with heroin has been the subject of at least two different studies with conflicting results found. An earlier study concluded that extremely high doses of pregabalin are consumed prior to or along with heroin in a deliberate attempt to enhance the effect of a dose of heroin (Grosshans et al., 2013). One could conclude from this result that in humans, pregabalin may both alter the level of tolerance to heroin respiratory depression in the user and also depress respiration inherently at such large doses. This would make the consumption of heroin and pregabalin extremely dangerous, with both an enhancement of heroin respiratory depression and a summation of pregabalin and heroin respiratory depression.

However, a later study (Lyndon et al., 2017), found that heroin users did not enjoy the ‘zombie’ like effect that larger doses of pregabalin were described as producing, and pregabalin was not consumed as a deliberate means to enhance the high from a dose of heroin. Both of these published works do however acknowledge the risk of pregabalin and heroin co-consumption by heroin users over a wide range of abuse pregabalin doses, though more work is needed to fully understand the temporal and method-based dangers of using both pregabalin and heroin.

Finally, whilst the results in this thesis would suggest, as with ethanol, that methadone provides relatively good protection from on top heroin and pregabalin use, one must consider again that methadone-maintained users are known to use heroin following perceived clearance of methadone in approximately 18 hr (Lyndon et al., 2017)In this context, the prolonged half-life of pregabalin in humans may result in appreciable levels of pregabalin out-lasting the protection of methadone treatment and subsequent use of heroin after the decline in methadone levels may still be affected by a pregabalin induced reduction of tolerance.

7.11 G-protein Vs GRK/Arrestin Signalling

7.11.1 Opioidergic Mechanisms

Investigating the mechanisms for opioidergic effects has centred on the mediation of opioidergic effects through either G-protein or GRK dependent signalling pathways (Williams et al., 2013). Previous work in a series of highly influential publications suggested that the desirable effect of opioids i.e. analgesia, was mediated through the G-protein signalling pathway and that the undesirable acute effect of opioids i.e. respiratory depression, constipation, euphoria (as a precedent to addiction), and tolerance; were mediated through the GRK dependent signalling pathway (Raehal et al., 2005, Bohn et al., 2002). Additionally, other publications have suggested for individual opioid agonists, rather than opioid agonists as a single entity, that tolerance is mediated through a GRK dependent signalling pathway, including fentanyl, oxycodone and methadone (Melief et al., 2010).

However, there is also a significant amount of research that suggests that different opioidergic effects are mediated differently by either G-protein or GRK signalling. Several publications have suggested that MOPr desensitization can be mediated by both signalling pathways dependent in the opioid agonist applied (Bailey et al., 2009b, Johnson et al., 2006). Similarly, opioid respiratory depression has been shown to be almost entirely attenuated by blockade of GIRK channels, downstream mediator of G-protein signalling (Montandon et al., 2016).

This section of the discussion considers the existing evidence for the acute actions of opioids being mediated through G-protein or GRK signalling, the development of tolerance being mediated through G-protein or GRK signalling and also discusses the development of G-protein biased opioid agonists and how they far they have come towards developing an improved therapeutic opioid.

7.11.2 Acute Actions of Opioids

The primary body of evidence that suggest the undesirable effects of opioid are mediated through a GRK signalling pathway was developed through characterisation of opioidergic effects in the arrestin-3 knock out (Arr-3 KO). These results were published across several papers that have since been highly cited and influential (Bohn et al., 2000, Bohn et al., 2002, Raehal et al., 2005). These papers found that that opioid induced respiratory depression and constipation were reduced (Raehal et al., 2005) and opioid antinociception was both enhanced and prolonged in the Arr-3 KO (Bohn et al., 2002). However, the acute rewarding property of morphine was found to be enhanced in the Arr-3 KO mice (Bohn et al., 2003).

Other work has not reproduced this enhancement of morphine reward in Arr-3 KO mice, though reward was also not attenuated (Urs and Caron, 2014). Opioid induced hyperlocomotion was also shown to be significantly reduced in Arr-3 KO mice in the same publication.

In contrast to these results, other publications have found that potentiation of G-protein activity, through the inhibition of multiple regulator of G-protein signalling (RGS) enzymes) is able to enhance opioid antinociception (Garzon et al., 2004, Zachariou et al., 2003). Additionally, inhibition of GIRK channels, which are activated by the dissociated $G_{\beta\gamma}$ subunit, by Tertiapin-Q significantly attenuated the ability of opioids to induced respiratory depression in rats (Montandon et al., 2016). Complimenting this result, this thesis demonstrated that no change in fentanyl respiratory depression or antinociception was observed when GRK2 was inhibited suggesting that perturbation of the GRK/arrestin signalling pathway does not ameliorate opioid respiratory depression.

There is a great deal of conflicting evidence regarding the acute effects of opioids and the means through which they mediate antinociception, respiratory depression, euphoria and constipation. Not only does this debate range across G-protein versus GRK dependent signalling, but also opioid agonists specific recruitment of these pathways.

7.11.3 Tolerance to Opioids

As well as understanding the mechanisms of acute opioidergic actions, the mechanisms through which opioids induce both tolerance and addiction are crucial in the search for improved pain therapeutics that do not decrease in efficacy over time or result in addiction and the consequent impact that has on a life.

Characterisation of the Arr-3 KO mouse found not only beneficial changes with the acute effects of opioids, but also demonstrated a decrease in morphine induced MOPr desensitization *in vitro* (Bohn et al., 2000) but also a delayed onset of opioid tolerance *in vivo* (Bohn et al., 2002). This result contrasts with later work which found tolerance to develop normally in the Arr-3 KO mouse, though using different opioid agonists (Koblish et al., 2017a). However, the overall conclusion taken forward by multiple research groups has been that opioid tolerance is induced by a GRK dependent signalling pathway (Manglik and Kruse, 2017, Schmid et al., 2017b, Bohn, 2017, Koblish et al., 2017b, Crombie et al., 2015).

This conclusion is not matched by multiple other publications that have demonstrated an important role for G-protein dependent signalling pathways in the development of tolerance. Morphine tolerance has been shown to be prevented by the knock-out of JNK (Melief et al., 2010), a signalling protein activated through G-protein dissociation. Expression of constitutively active PKCα resulted in significantly enhanced tolerance to morphine antinociception and respiratory depression (Lin et al., 2012).

Indeed, at a receptor level, PKC inhibition is able to prevent desensitization in an opioid agonist specific manner (Bailey et al., 2009a, Bailey et al., 2009b), suggesting that different opioid agonists induce desensitization and potentially tolerance by G-protein or GRK dependent mechanisms. Characterisation of intrinsic efficacy for both G-protein and arrestin recruitment by opioid agonists suggests that a single modality of tolerance induction is unlikely given the difference in relative signalling efficacy (McPherson et al., 2010).

If one were to consider the body of work presenting GRK signalling as the prime mediator of opioid tolerance, then manipulation of G-protein dependent pathways, such as inhibition of PKC, should remain ineffective against tolerance for all four agonists, as induced tolerance would be driven and maintained by an arrestin dependent pathway.

As PKC inhibition, or the absence of PKCα, profoundly reverse or inhibits the development of morphine induced tolerance and PKC inhibition also reverse oxycodone induced tolerance then this would suggest that induction of maintenance of tolerance induced by these two opioid agonists with low intrinsic efficacy for arrestin signalling, is in fact mediated through G-protein signalling rather than mediated through arrestin signalling.

Conversely, both methadone and fentanyl induced tolerance, with each agonist possessing high intrinsic efficacy for arrestin signalling, remained unaffected by PKC inhibition. Indeed, antinociceptive tolerance to fentanyl was prevented by inhibition of GRK2/3, which are required for mediating arrestin recruitment and further downstream signalling.

These results suggest that no broad umbrella can be cast over opioid agonists regarding the development of tolerance at the MOPr, but rather that each agonist must be considered independently with regard to the specific signalling profile possessed by each agonist.

7.11.4 G-protein Biased Opioid Agonists

The characterisation of morphine and other opioids in the arrestin-3 knock out mouse as being therapeutically improved (Bohn et al., 2002, Raehal et al., 2005), forwarded the hypothesis that developing a MOPr specific opioid agonist that displayed a bias in signalling towards the G-protein pathway would provide an opioid that had both an improved analgesic output and a decreased propensity to cause side effects such as potentially lethal respiratory depression.

This hypothesis led to the pursuing of opioid agonists with a signalling profile biased to G-protein activation (Manglik and Kruse, 2017, Schmid et al., 2017b, Bohn, 2017, Koblish et al., 2017b, Crombie et al., 2015), attempting to develop an opioid devoid of both tolerance and respiratory depression.

The pharmaceutical company Trevena developed a flagship biased opioid agonist labelled TRV130 and later marketed as Oliceridine (Schneider et al., 2016, Singla et al., 2017). In vitro data suggested that it was weakly biased towards G-protein and in vivo data suggested that it had a decreased propensity to induce respiratory depression yet still have significant antinociceptive efficacy (DeWire et al., 2013b). Subsequent human trials have demonstrated that TRV130 provides a marginal improvement in the induction of respiratory depression and nausea (Singla et al., 2017), however TRV130 has a significantly shorter half-life than morphine and so requires repeat dosing to achieve the same consistent level of analgesia in humans (Singla et al., 2017). Indeed, TRV130 was also found to be just as rewarding in pre-clinical studies as morphine (Altarifi et al., 2017), with a worrying lack of data recorded on the liking of TRV130 by human patients to assess potential abuse liability.

Another opioid agonist described as G-protein biased was developed and labelled PZM21 (Manglik et al., 2016b) with in vivo studies showed that it had no rewarding properties, was not constipating, provided significant antinociception, yet crucially was devoid of respiratory depression. However, subsequent research has suggested that PZM21 does in fact depress respiration (Hill et al., 2018).

There are however, other opioid agonists that are described as biased that have promising therapeutic applications. These include a series of pure MOPr agonists that are G-protein biased (Schmid et al., 2017b) and there is also an agonist known as mitragynine pseudoindoxyl (Varadi et al., 2016) that has G-protein biased agonism at the MOPr but acts as an antagonist at the DOPr.

The pursuit of a G-protein biased opioid agonist may however, be coloured by an over simplified view of receptor signalling at the level of the MOPr, and consideration for activating both pathways at the same time, to multiple degrees of efficacy must be considered. This would reconcile the two strands of research which show clear importance for both the G-protein and GRK dependent signalling pathways at the MOPr.

7.11.5 Developing the Two-Pathway Signalling Profile

A large amount of scientific work surrounding opioid induced tolerance, and the subsequent pursuit of biased opioid agonists centres around an "A-or-B" logic, wherein any given action of opioid agonist is adjudged to be mediated through pathway A (i.e. G-protein signalling) or pathway B (i.e. arrestin signalling) (Manglik and Kruse, 2017, Schmid et al., 2017b, Bohn, 2017, Koblish et al., 2017b, Crombie et al., 2015). However, this model does not consider the dual recruitment of both pathways that might also culminate in the same end point, that is to say that tolerance to a given opioid agonist is generated by "A-and-B" with G-protein and arrestin signalling contributing to a single measurable output such as tolerance.

Alternatively, recent work presented at the International Narcotics Research Conference by Macdonald Christie, suggests that a more desirable therapeutic index from an opioid might be developed by consideration of the intrinsic G-protein efficacy of an opioid agonist, with low partial efficacy for G-protein activation providing decreased opioid respiratory depression. Indeed, when one considers the currently published G-protein biased opioid agonists, they do share a common trait of having a lower maximum of G-protein activation (i.e. partial agonism) compared to canonical agonists.

7.12 Future Direction

Despite extensive research into the mechanisms that underlie the development of opioid tolerance, many questions still remain. This final discussion will briefly consider the short, medium and long terms goals of opioid research. Of particularly pressing concern is the rampant use of fentanyl on the streets of the USA and the potency and availability of fentanyl must permeate all concerns regarding opioid overdose if a sufficiently effective response to this crisis is to be developed (Compton et al., 2016).

7.12.1 Short-Term Goals

I. Investigate the potential of fentanyl to breakthrough morphine tolerance
    a. Fentanyl is not thought to be a first-choice opioid of abuse for most opioid dependent users, but consumption of fentanyl is considered accidental due to the cutting of other opioids with fentanyl (Compton et al., 2016). In these cases, existing tolerance will have been induced by another opioid and fentanyl, due to its unique pharmacological presentation, may be able to breakthrough well established tolerance and induce fatal respiratory depression.

II. Investigate the underlying reason for fentanyl resistance to naloxone antagonism
    a. Conventional pharmacology predicts that naloxone would reverse equipotent doses of morphine and fentanyl equally. Given that this is not the case, wit is important yo determine the unique component of fentanyl pharmacology that grants its resistance to antagonism. Molecular dynamic simulation of fentanyl binding to the MOPr may grant insight into unique residue interactions in the orthosteric binding pocket or indeed present a unique non-orthosteric binding pocket for fentanyl that is inaccessible to naloxone.
    b. The lipophilicity of fentanyl may be able to produce micro domain concentrations of fentanyl in the adjacent lipid bilayer that in conjunction with a unique route of binding or a unique binding site would allow a fast on-off rebinding rate of fentanyl preventing competitive antagonism by naloxone. The difference in bulk phase versus membrane phase concentrations of agonists has previously been described (Gherbi et al., 2018).

III. Investigating a direct link between ethanol/acetaldehyde and PKC inhibition

a. PKC translocation from the cytosol to the plasma membrane occurs on PKC activation. Therefore, PKC translocation as an indicator of PKC activity can be assessed using sub-cellular fractionation. This could be achieved with PMA as a positive control to investigate inhibition of translocation by PKC inhibitors and ethanol/acetaldehyde as well as pregabalin. Translocation could also be measured in response to varying opioid agonists.

7.12.2 Medium-Term Goals

I. Improved specificity of signalling pathway modulation

a. Past research into the relevance of G-protein or GRK signalling with regards to opioidergic effects have suffered from a relative lack of clarity due to the contemporary tools available. Work performed in knock-out mice may well have compensatory mechanisms through development that render the results difficult to interpret (El-Brolosy and Stainier, 2017). Conditional knock-in mice, coupled with the use of more specific inhibitors such as 14as a structurally unrelated to compound 101 GRK inhibitor (Waldschmidt et al., 2017), could be used to further investigate the mechanism by which opioids mediate physiological changes.

b. Conditional knock-in or conditional knock-out mouse lines are now able to be developed through the system of Cre-loxp (Feil et al., 2009). Cre-loxp allows specific gene coding to express normally throughout development prior to the administration of Cre, which selectively alters expression of a given protein in specifically targeted neuronal sites. Given that the rate of tolerance development to differing opioidergic effects such as antinociception and respiratory depression are thought to be different, this would allow brain nuclei specific knock-in and-out of signalling molecules to assess their impact on opioid tolerance.

c. Key brain regions for the regulation of respiration such as the Kölliker fuse, bötzinger, pre-bötzinger and post-inspiratory complex nuclei (Lalley et al., 2014a, Lalley et al., 2014b) would be exemplary targets to reduce the expression of arrestin and PKC isoforms by Cre-loxp editing and subsequently assess the impact of these changes in expression on the development of opioid tolerance to respiratory depression. This would preserve normal network development and receptor organisation and trafficking prior to manipulation, removing some degree of uncertainty on the validity of the results obtained. This technique could also be employed to investigate the development of tolerance to opioid antinociception by targeting separate brain and spinal regions such as the periaqueductal grey and dorsal root ganglion neurones.

II. Improved spatial resolution of receptor activation

a. site-specific activation of opioid receptors through a so called "*caged-ligand*". Caged ligands are inactive until activated by specific wavelengths of light (Tadevosyan et al., 2016). The development of a caged opioid agonist would allow systemic administration of an inactive opioid agonist, following which site specific opto-stimulation would "uncage" the agonist and allow it to bind to opioid receptors. The degree of opto-illumination can be controlled very precisely with modern fibreoptic diodes, thus preventing overt diffusion of the agonist to undesired brain regions. This would allow the examination of specific opioid agonist application to one brain nuclei, wherein the contribution of that nuclei to tolerance as a whole could be examined. For example, there is great discussion on the relative importance of different respiratory nuclei on the control of respiration, selective application of opioid to a single nucleus would illuminate the degree to which that nuclei influences respiratory rhythm, depth of respiration or rate of respiration. Certain respiratory nuclei, such as the Kölliker Fuse have also been suggested to lack desensitization of the MOPr. This approach would allow that to be placed in an in vivo context where tolerance may not develop on repeat discreet administration of an opioid agonist to that nucleus.

III. Obtain more recent data on polydrug abuse in humans

a. Whilst ethanol has been a notable additional drug of abuse with opioid for decades, the emergence of gabapentoids as abusable co-drugs with opioids clarifies that the drug-taking landscape is constantly changing and there is a need to ensure potential drug-drug interactions are not missed through lack of engagement with opioid users; though this has not historically been a problem. There is however a difficulty in gaining consensus data due to the differences in national and international methods of data recording, with different drugs tested for during post-mortem analysis.

7.12.3 Long-Term Goals

I. Investigate respiratory depression and euphoria in opioid users in a longitudinal study

a. Recruiting a cohort of opioid users over a significant period of time with measurement of dose, route of administration, respiratory depression by human whole-body plethysmography and a subjective measure of euphoria would allow more conclusive results on the development of tolerance in humans and the relative rate of their occurrence for the primary determining opioidergic effects in human opioid abusers.

b. A record through self-assessment or subject sampling would also allow polydrug abuse to be monitored throughout the study and allow assessment of variations in tolerance or drug potency based on additionally abused drugs.

II. Investigate the role of chronic pain in opioid tolerance

a. Chronic pain conditions are known to have aberrant signalling systems often in the absence of actual tissue damage and are often co-morbid with hyperalgesia and/or allodynia. Given the relative lack of efficacy of opioids to provide analgesia in chronic pain patients, an investigation into the cellular mechanisms underlying chronic pain may reveal a commonality in the pathways of chronic pain and opioid tolerance, such that the underlying opioidergic tone in chronic pain patients presents a baseline tolerance rendering exogenous opioids ineffective in producing pain relief.

7.13 Conclusion

The past two decades have seen a huge advancement in the understanding of mechanisms through which opioids function. Though there is still a division within the community of scientists investigating opioids regarding which signalling pathway is responsible for individual opioidergic effects, the field of vision has narrowed, and the specificity of research has enabled the minutiae of these pathways to be scrutinised. Inevitably, there will be a middle ground that incorporates the evidence from both sides in what is a complex and nuanced signalling system, the understanding of which is vital for both the development of better analgesics and the better treatment of the opioid addicted.

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